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Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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13657

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8
Construction and Analysis of cDNA Libraries

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The process of enzymatic conversion of poly(A)$^+$ mRNA to double-stranded DNA and the insertion of this DNA into prokaryotic vectors has become a fundamental tool of eukaryotic molecular biology. Since the first clones of complementary DNA (cDNA) were obtained in the mid-1970s, many different methods have been developed to increase the efficiency of synthesis of double-stranded cDNA and many improvements have been made to the vector systems. In the first part of this chapter, we describe some of these developments, pointing out their advantages and disadvantages. In the second part, we present in detail the procedure that is now most commonly used: synthesis of the first strand of cDNA with reverse transcriptase, replacement synthesis of the second strand of cDNA (using RNAase H and the Klenow fragment of *Escherichia coli* DNA polymerase I), addition of synthetic linkers, and finally, cloning into a bacteriophage λ vector.

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Strategies for cDNA Cloning

The synthesis and cloning of cDNA are still not easy, but as a consequence of a wide range of technical and theoretical advances, cDNA cloning is now well within the range of any competent laboratory. Comprehensive cDNA libraries can be routinely established from small quantities of mRNA, and a variety of reliable methods are available to identify cDNA clones corresponding to extremely rare species of mRNA. As the enzymatic reactions used to synthesize cDNA have improved, the sizes of cloned cDNAs have increased, and it is often possible to isolate cloned full-length cDNAs corresponding to large mRNAs.

Before embarking on the synthesis and cloning of cDNA, however, it is essential to consider carefully which methods, vectors, and screening procedures offer the best chance of success. Which combination of these factors is most appropriate for a particular cloning problem depends on the information and reagents that are available, for example, whether the sequence of the protein is known or can be obtained or whether suitable antibodies are available.

PREPARATION OF mRNA FOR cDNA CLONING

Source of the mRNA

Clearly, the higher the concentration of the sequences of interest in the starting mRNA, the easier the task of isolating relevant cDNA clones becomes. It is therefore worthwhile investing some effort to make sure that the richest source of mRNA available is being used. This can be achieved, for example, by using immunoprecipitation to measure the amount of the protein of interest that is synthesized in cell-free systems by mRNAs prepared from different cell lines or tissues. In addition, some workers have found ways to increase the concentration of the relevant mRNA by using drugs to select cell lines that overexpress particular proteins. For example, Luskey et al. (1983) describe the use of the drug compactin to develop a line of Chinese hamster ovary cells that have undergone a 15-fold increase in the number of gene copies and a much greater increase in the amount of mRNA coding for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase). Other workers have taken advantage of the observation that treatment of infected cells with inhibitors of protein synthesis causes extended transcription of the early genes of mammalian DNA viruses (e.g., Persson et al. 1981). In these and a number of similar cases, increased abundance of specific classes of rare mRNAs greatly facilitated their subsequent cloning.

Whenever possible, estimates should be obtained of the frequency with which the mRNA of interest occurs in the starting preparation. As discussed below, this frequency not only determines the size of the cDNA library that is needed, but it may also influence the method used to screen it.

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Integrity of the mRNA

Since the cDNA library cannot be better than the mRNA from which it is derived, it is important to check the integrity of the preparation of mRNA before it is used as the template for synthesis of the first strand of cDNA. The following tests are commonly used:

- *The ability of the mRNA to direct the synthesis of high-molecular-weight proteins in cell-free translation systems derived from reticulocytes* (see Chapter 18). Bulk mRNA purified from mammalian cells should code for a large number of polypeptides with molecular weights ranging from less than 10,000 to greater than 100,000. An autoradiograph of the products of translation of an undegraded preparation of mammalian mRNA is shown in Figure 8.1.
- *The ability of the mRNA to direct the synthesis of the polypeptide of interest in a cell-free system derived from reticulocytes.* An aliquot of the translation products of the bulk mRNA is analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. After autoradiography, the radioactive band can be excised from the gel and counted in a scintillation counter. The proportion of the total radioactivity contained in the band is an estimate of the relative concentration of the mRNA of interest.
- *The size of the mRNA.* If a sufficient quantity of mRNA is available, the mRNA should be fractionated by gel electrophoresis and stained with ethidium bromide (see Chapter 7). The mRNA should appear as a smear between approximately 500 bases and 8 kb. The bulk of the mRNA should lie between 1.5 and 2 kb.

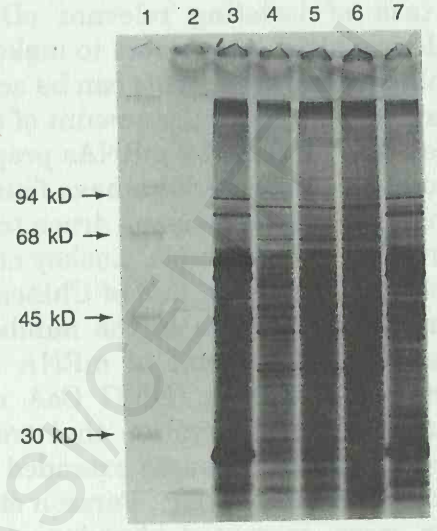

FIGURE 8.1
Translation of mRNAs in reticulocyte lysates. Radiolabeled polypeptides, generated by translation of mammalian mRNAs in a lysate of reticulocytes, are displayed on an autoradiograph of an SDS-polyacrylamide gel. (*Lane 1*) Molecular weight markers; (*lane 2*) no mRNA added to the translation reaction; (*lanes 3 and 7*) 8 μg of total mRNA extracted from CV-1 cells infected with A/Japan strain of influenza virus; (*lanes 4, 5, and 6*) 0.25, 0.5, and 1.0 μg, respectively, of poly(A)$^+$ RNA purified from the Bowes line of human melanoma cells.

- *The ability of the bulk mRNA preparation to direct the synthesis of long molecules of first-strand cDNA.* The radioactive cDNA synthesized in a pilot reaction (see page 8.61) is analyzed by electrophoresis through an alkaline 1% agarose gel, using as markers end-labeled fragments of DNA that range in size from 500 bases to 8 kb. cDNA synthesized from mammalian mRNA should run as a continuous smear from approximately 500 bases to more than 5 kb (Figure 8.2). The bulk of the radioactivity should lie between 1.5 kb and 2 kb, and there should be no specific bands of cDNA visible unless the mRNA was prepared from cells that express large quantities of specific mRNAs. Finally, the amount of radioactivity incorporated into the first strand of cDNA should be stimulated at least 20-fold by oligo$(dT)_{12-18}$ primers. Efficient cDNA synthesis in the absence of added primer is a sign that the mRNA preparation may be contaminated with fragments of DNA or RNA that can bind to mRNA at random sites and serve as primers. If necessary, these fragments can be removed by denaturing the mRNA (100°C for 30 seconds in water), cooling quickly in ice water, and immediately selecting poly$(A)^+$ RNA by chromatography on oligo(dT)-cellulose.

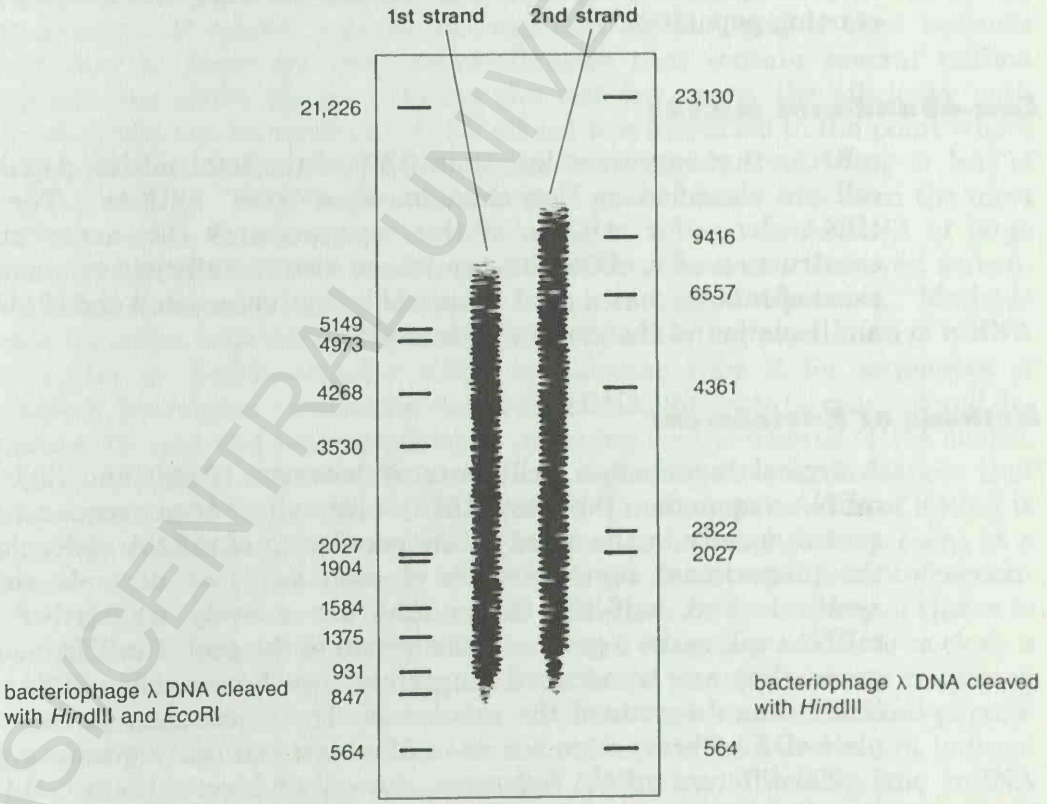

FIGURE 8.2
Alkaline agarose gel electrophoresis of first and second strands of cDNA synthesized from poly$(A)^+$ RNA extracted from mammalian cells. Undegraded mRNA should yield first-strand cDNA molecules that range in size from approximately 700 nucleotides to more than 8000 nucleotides in length. Second-strand cDNA made by self-priming should range in size from approximately 900 nucleotides to more than 10,000 nucleotides in length.

Abundant mRNAs

Initially, cDNA cloning was used to obtain copies of abundant mRNAs such as those encoding globin, immunoglobulins, and ovalbumin. In these cases, the RNA species of interest constitutes as much as 50–90% of the total poly(A)$^+$ cytoplasmic RNA isolated from specific types of differentiated cells. Consequently, no further purification of the particular mRNA is required before double-stranded cDNA is synthesized and cloned. The desired cDNA clones can easily be identified by nucleic acid hybridization. The probes consist either of ^{32}P-labeled single-stranded cDNA synthesized in vitro by reverse transcriptase, using as the template mRNA preparations that are rich in the sequences of interest, or of mRNA that has been partially fragmented by limited alkaline hydrolysis and end-labeled by phosphorylation. As a good approximation, the mRNA sequences of interest will be represented in both the probe and the cloned double-stranded cDNAs in proportion to their abundances in the original preparation of mRNA. In cases such as globin, immunoglobulins, and ovalbumin, the chances are high that any colony hybridizing strongly to the probe will contain the desired DNA sequences. Although used extensively in the early days of cDNA cloning, this method no longer finds wide application, since few systems remain in which interesting uncloned mRNAs represent a sufficiently high proportion of the starting population.

Low-abundance mRNAs

mRNAs that represent less than 0.5% of the total mRNA population of the cell are classified as "low-abundance" or "rare" mRNAs. The isolation of cDNA clones for mRNAs of this type presents two major problems: (1) construction of a cDNA library whose size is sufficient to ensure that the clone of interest has a good chance of being represented and (2) identification and isolation of the clone(s) of interest.

Methods of Enrichment

A typical mammalian cell contains between 10,000 and 30,000 different mRNA sequences (Davidson 1976). Not all of these sequences are represented equally in the steady-state population of mRNA molecules. Instead, the proportional representation of each sequence depends on its rate of synthesis and half-life: Genes that are actively transcribed into stable mRNAs will make a greater contribution to the pool of mRNA molecules than genes that are transcribed sluggishly into less stable mRNAs. Williams (1981) has determined the number of clones necessary to construct a complete cDNA library from a human fibroblast cell that contains approximately 12,000 different mRNA sequences. Low-abundance mRNAs (<14 copies/cell) constitute approximately 30% of the mRNA, and there are about 11,000 different mRNAs belonging to this class. The minimum number of cDNA clones required to obtain a complete representation of mRNAs of this class is therefore $11{,}000/0.30 \cong 37{,}000$. Of course, because of sampling variation and/or preferential cloning of certain sequences, a much larger number of recombinants must be obtained to increase the chances that any given clone

will be represented in the library. The number of clones required to achieve a given probability that a low-abundance mRNA will be present in a cDNA library is

$$N = \frac{\ln(1-P)}{\ln(1-1/n)}$$

where N = the number of clones required, P = the probability desired (usually 0.99), and $1/n$ = the fractional proportion of the total mRNA that is represented by a single type of rare mRNA.

Therefore, to achieve a 99% probability of obtaining a cDNA clone of an mRNA present in human fibroblasts at a frequency of approximately 14 molecules/cell:

$P = 0.99$
$1/n = 1/37{,}000$
$N = 170{,}000$

Unfortunately, many mRNAs of interest are present at even lower levels (1 molecule/cell is not unusual [Toole et al. 1984; Wood et al. 1984]). Furthermore, it is often necessary to clone cDNAs from populations of mRNAs isolated from tissues that consist of several cell types. In such cases, the frequency at which the sequences of interest are represented in the initial preparation of mRNA may be reduced still further, and it then becomes necessary to construct and screen libraries that contain several million independent cDNA clones. During the last few years, the efficiency with which cDNA can be synthesized and cloned has increased to the point where cDNA libraries of this size can be generated routinely from 10 μg or less of poly(A)$^+$ mRNA. In principle, there is no a priori reason why even the most difficult cDNA clones—those corresponding to a very rare mRNA of large size—cannot be identified in such comprehensive libraries. However, screening large numbers of cDNA clones is both tedious and expensive. Methods have therefore been devised to enrich either the starting population of mRNA molecules or double-stranded cDNA synthesized from it for sequences of interest. Enrichment allows the size of the cDNA library to be reduced and decreases the cost and labor involved in screening for the desired cDNA clones.

It is difficult to offer specific guidelines regarding the circumstances that require enrichment procedures. As a rule of thumb, fractionation of mRNA is probably unnecessary if the cDNA of interest is expected to be present at a frequency ≥ 1 in 10^6 in a library of cDNA clones synthesized from unfractionated mRNA. Enrichment becomes more attractive as the number of clones to be screened increases above one million. When designing a scheme to clone a specific cDNA, it is therefore important to know the approximate frequency with which the mRNA of interest occurs in the bulk, unfractionated population of mRNA molecules. In the absence of nucleic acid probes, an indirect method must be used to measure this frequency. Usually, the mRNA preparation is translated in a cell-free system and the total amount of radioactivity incorporated into protein is measured. The polypeptide of interest is then immunoprecipitated and identified by electrophoresis through an SDS-polyacrylamide gel. The amount of radioactivity in the excised band is then measured and used to calculate the proportion of the total counts that have been incorporated into the protein of interest. This proportion is taken

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as a measure of the frequency with which the mRNA occurs in the bulk population. Despite its obvious limitations, this method usually yields estimates that are sufficiently reliable to allow rational schemes for cDNA cloning to be devised.

Clearly, fractionation works best for mRNAs that are much larger or smaller in size than the bulk mRNA of the cell. The modal size of the mRNA population extracted from most types of mammalian cells is approximately 1.8 kb, and mRNAs smaller in size than 700 bases or larger than 4 kb can be enriched at least tenfold by a single round of density gradient centrifugation carried out under denaturing conditions. However, it is important to remember that it is not possible to predict with certainty the size of an mRNA from the size of a protein for which it codes. There is considerable variation in the sizes of the untranslated regions of mRNAs (particularly the 3′ untranslated regions); many proteins purified from cells are cleavage products of larger precursors and many undergo extensive posttranslational modification. However, the size of the unmodified polypeptide chain provides a minimal estimate of the size of the mRNA: 10,000 daltons of an average polypeptide is encoded by approximately 280 bases of mRNA.

FRACTIONATION OF mRNA BY SIZE

The simplest method to enrich preparations of mRNA for sequences of interest is to fractionate them according to size. Electrophoresis through agarose gels gives the best separation of molecules of mRNA of different sizes, but the recovery of RNA from gel slices is generally poor. Sedimentation through sucrose gradients formed in nondenaturing solvents results in good recovery, but the presence of secondary structure in the RNA often confounds effective fractionation. The method of choice, therefore, is sucrose gradient centrifugation in the presence of an agent, such as methylmercuric hydroxide, that denatures secondary structure in RNA (Schweinfest et al. 1982) (for experimental protocol, see Chapter 7, page 7.35). Each fraction is then assayed for the presence of the mRNA that codes for the relevant polypeptide. Typically, an aliquot of the RNA in each fraction is translated in a cell-free system and the resulting polypeptides are analyzed by immunoprecipitation and electrophoresis through polyacrylamide gels. Alternatively, aliquots are injected into *Xenopus* oocytes (for review, see Melton 1987) and the resulting products are assayed either for biological activity or by immunoprecipitation and gel electrophoresis. The fraction that directs the synthesis of the greatest amount of the polypeptide product is then used as the starting material for construction of a cDNA library.

FRACTIONATION OF cDNA

Until a few years ago, fractionation of mRNA was the method of choice for cloning of mRNAs that code for large proteins (e.g., rat skeletal muscle tropomyosin [Medford et al. 1980] and chick creatine kinase [Schweinfest et al. 1982]). However, as methods for the synthesis of cDNA have improved, fractionation of double-stranded cDNA has become a more practical alternative, and there are now many examples of extremely large cDNAs that have been cloned by fractionating cDNA rather than the mRNA from which it was

copied (e.g., human factor VIII:C [Toole et al. 1984; Wood et al. 1984] and human sucrase-isomaltase [Hunziker et al. 1986]). Fractionation of cDNA has major advantages: DNA is less susceptible than mRNA to degradation by contaminating nucleases; it can be fractionated more accurately by electrophoresis through agarose gels; and, finally, since the fractionation can be carried out at a late stage during the cDNA cloning protocol, the chances of subsequent mishaps are reduced and the probability of obtaining a full-length clone of cDNA is increased. Fractionation is usually carried out after all of the enzymatic reactions involved in cDNA synthesis have been completed and just before the cDNA is inserted into a vector. In the detailed protocol described later in this chapter, fractionation is carried out after synthetic linkers, added to the termini of double-stranded cDNA, have been digested with a restriction enzyme. The cDNA is fractionated by electrophoresis through an agarose gel of appropriate porosity, using markers whose sizes are known accurately. Molecules of the desired size are recovered and inserted into the vector.

IMMUNOLOGICAL PURIFICATION OF POLYSOMES

An alternative method of enrichment involves the use of antibodies to purify polysomes that are synthesizing the polypeptide of interest. The technique described originally (Palacios et al. 1972; Schechter 1973), which involved the immunoprecipitation of polysomes, worked well for mRNAs encoding abundantly synthesized proteins such as albumin and immunoglobulin, although attempts to apply the method to mRNAs of lesser abundance were generally disappointing. However, the more recent use of immunoaffinity columns (Schutz et al. 1977) and protein A–Sepharose columns (Shapiro and Young 1981) has led to a resurgence of the technique. For example, Korman et al. (1982) used a monoclonal antibody directed against the heavy chain of the human HLA-DR histocompatibility antigen to bind polysomes synthesizing the nascent protein to protein A–Sepharose columns. The polysomes were then dissociated with EDTA and the mRNA isolated by oligo(dT) chromatography. The immunoaffinity-purified mRNA, which represented only 0.01–0.05% of the total mRNA, was used both to prepare cDNA probes and to construct cDNA clones. Using similar methods with polyclonal antisera, Kraus and Rosenberg (1982) obtained a 6300-fold purification of the mRNA that codes for rat liver cystathionine β-synthase and Russell et al. (1983) isolated cDNA clones for the bovine low-density-lipoprotein receptor, whose mRNA is present at approximately 80 copies per cell in bovine adrenal cells.

Although a powerful technique, immunoaffinity purification of polysomes cannot be applied universally. First, it clearly will not work unless a reliable source of material is available from which to isolate functional polysomes. This is not always possible, especially when the starting material is a tissue or organ that is not commonly available. Second, it has not yet been shown to work for mRNAs that are extremely rare (1 molecule/cell or less). Furthermore, the success of the method depends entirely on the specificity, avidity, and type of the particular antibody, and it is not always possible to translate the results obtained with one antibody directly to another. Finally, the method requires the use of relatively large quantities of antibody. Partly

because of these difficulties, immunoaffinity purification of polysomes has been superseded by development of cDNA cloning vectors (e.g., λgt11 and λgt18–23) that allow the direct isolation of cDNA clones that code for specific antigens. Whether or not the method is used extensively in the future will depend on improving its sensitivity to the point where it provides significant enrichment of polysomes carrying extremely rare mRNAs.

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SYNTHESIS OF THE FIRST STRAND OF cDNA

All methods for synthesis of the first strand of cDNA use the enzyme RNA-dependent DNA polymerase (reverse transcriptase) to catalyze the reaction (Figure 8.3). Two different forms of reverse transcriptase are available commercially: avian reverse transcriptase, which is purified from particles of an avian retrovirus, and murine reverse transcriptase, which is isolated from a strain of *E. coli* that expresses a cloned copy of the reverse transcriptase gene of the Moloney murine leukemia virus. Commercial preparations of both forms of the enzyme are now reasonably free of contaminating RNAase, which, in the past, had greatly affected the size of the cDNA. However, preparations of the avian enzyme can be contaminated by an endonuclease that cleaves DNA, although this is less of a problem today than it was in the early 1980s. The avian enzyme consists of two polypeptide subunits that carry several enzymatic activities—RNA-dependent synthesis of DNA, DNA-dependent synthesis of DNA, and endonucleolytic attack on the RNA moiety of DNA:RNA hybrids followed by processive exonucleolytic removal of rNTPs (RNAase H activity). The murine enzyme consists of a single polypeptide subunit that carries out both RNA- and DNA-dependent synthesis of DNA but has a reduced capacity to degrade RNA in DNA:RNA hybrids, and preparations of murine reverse transcriptase lack the con-

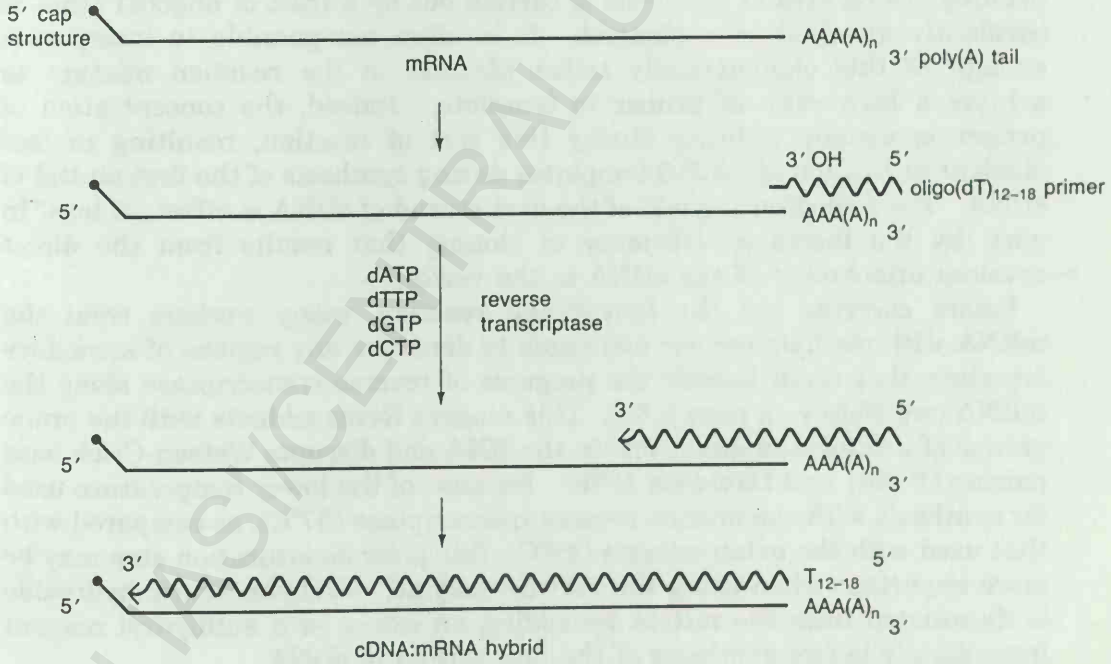

FIGURE 8.3
Synthesis of the first strand of cDNA using an oligo(dT) primer and reverse transcriptase.

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taminating endonuclease activity (Gerard 1983; Kotewicz et al. 1988). The avian enzyme available from many manufacturers is adequate for routine construction of cDNA libraries. However, the high level of RNAase H activity of the avian enzyme tends both to suppress the yield of cDNA and to restrict its length. At the beginning of the reaction, the hybrids formed between the template mRNA and the primer are a substrate for RNAase H. Thus, at the beginning of cDNA synthesis, there is a competition between degradation of the mRNA and initiation of DNA synthesis. In addition, RNAase H can cleave the RNA template near the 3′-hydroxyl terminus of the growing DNA strand if the polymerase pauses during synthesis (Kotewicz et al. 1988). Thus, the murine enzyme, with its weaker complement of endogenous degradative activities, is a safer choice when attempting to obtain full-length cDNA copies of mRNAs larger than 2–3 kb in length. The murine and avian enzymes display different pH, salt, and temperature optima for the synthesis of cDNA from RNA templates, and it is therefore important to modify the conditions for first-strand synthesis accordingly. (See Chapter 5 for a more detailed discussion of reverse transcriptases.)

Like other DNA polymerases, avian and murine reverse transcriptases require a primer to initiate synthesis of DNA. For cloning of cDNAs, the most frequently used primer is oligo(dT) 12–18 nucleotides in length, which binds to the poly(A) tract at the 3′ terminus of eukaryotic cellular mRNA molecules. The primer is added to the reaction mixture in large molar excess so that each molecule of mRNA binds several molecules of oligo$(dT)_{12-18}$. Priming of cDNA synthesis probably begins from the most proximal of these bound primers and is very efficient. However, in some cloning procedures (see, e.g., Okayama and Berg 1982, 1983 and Figure 8.6 on pages 8.18–8.19), priming of first-strand synthesis is carried out by a tract of oligo(dT) that is covalently attached to a plasmid. It is often not possible to incorporate enough of this oligomerically tailed plasmid in the reaction mixture to achieve a high ratio of primer to template. Indeed, the concentration of primer is usually limiting during this sort of reaction, resulting in less efficient utilization of mRNA templates during synthesis of the first strand of cDNA. The reduction in yield of the first strand of cDNA is offset, at least in part, by the increased efficiency of cloning that results from the direct covalent attachment of the cDNA to the vector.

Before carrying out the first-strand reaction, many workers treat the mRNA with methylmercuric hydroxide to denature any regions of secondary structure that could impede the progress of reverse transcriptase along the mRNA (see Note v on page 8.62). This reagent forms adducts with the imino groups of uridine and guanosine in the RNA and disrupts Watson-Crick base pairing (Bailey and Davidson 1976). Because of the lower temperature used for synthesis with the murine reverse transcriptase (37°C), as compared with that used with the avian enzyme (42°C), this prior denaturation step may be more important when using the murine enzyme. Methylmercuric hydroxide is dissociated from the mRNA by adding an excess of a sulfhydryl reagent immediately before synthesis of the first strand of cDNA.

To minimize the deleterious effects of RNAases that may contaminate the solutions or reagents used for the synthesis of the first strand of cDNA, a potent protein inhibitor of RNAase, purified from human placenta, is usually

included in the reaction (de Martynoff et al. 1980). RNAase binds noncovalently to this protein to form an enzymatically inactive complex consisting of equimolar quantities of RNAase and inhibitor (Blackburn and Jailkhani 1979).

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SYNTHESIS OF THE SECOND STRAND OF cDNA

Self-priming

For reasons that are not fully understood, the 3′ termini of single-stranded cDNAs are capable of forming hairpin structures that can be used to prime the synthesis of the second strand of cDNA by the Klenow fragment of *E. coli* DNA polymerase I or reverse transcriptase (Figure 8.4). To allow these structures to form, it is necessary to denature the DNA:RNA hybrid molecules that are the products of the first-strand reaction. For many years, there

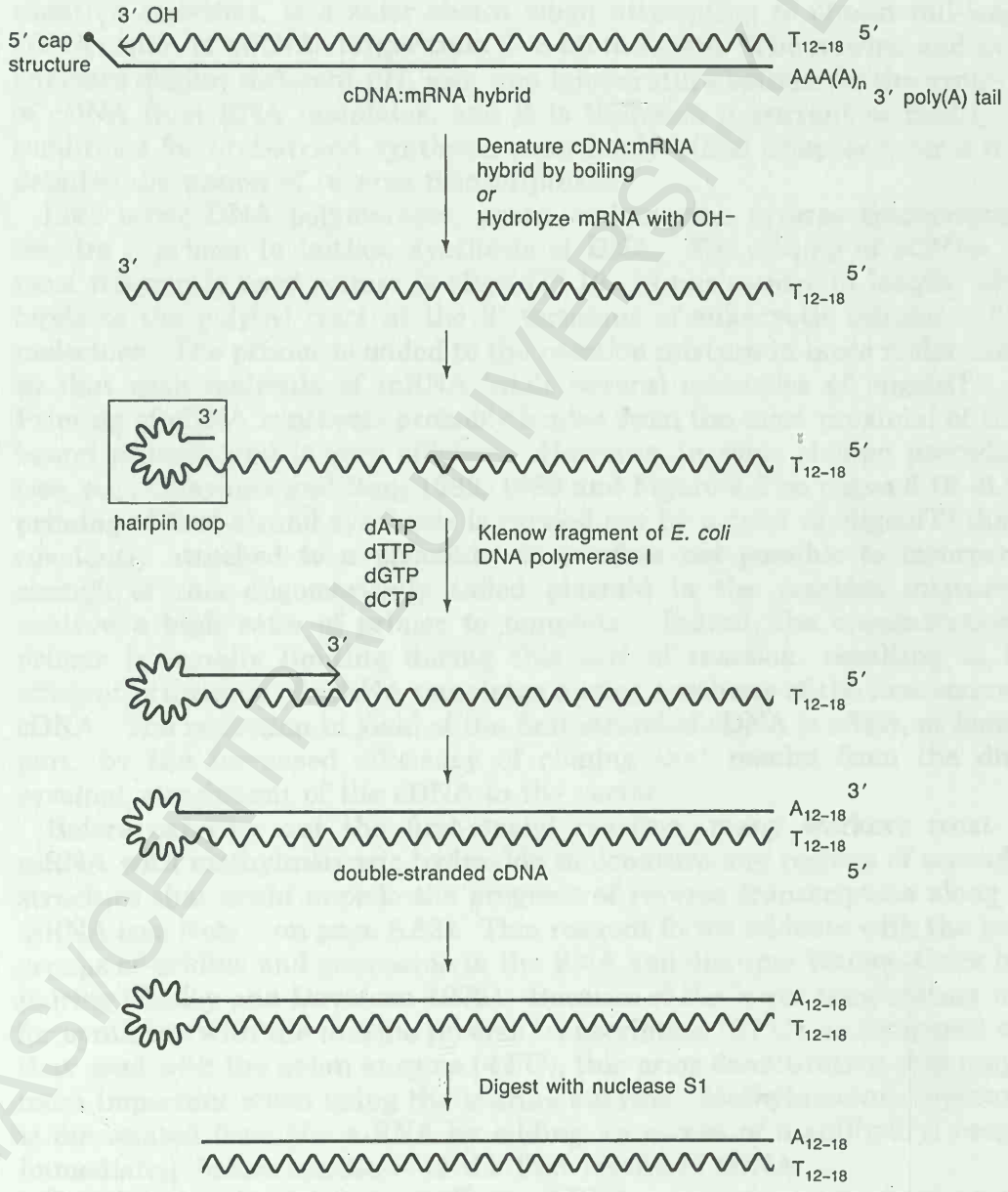

FIGURE 8.4
Synthesis of double-stranded cDNA by the self-priming method.

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was no effective way other than self-priming to synthesize the second strand of cDNA, and almost all clones of cDNA made before 1982 were obtained using this enzymatic reaction (see, e.g., Efstratiadis et al. 1976; Higuchi et al. 1976; Maniatis et al. 1976; Rougeon and Mach 1976). The product of the self-primed synthesis of the second strand was a double-stranded cDNA molecule closed at the terminus corresponding to the 5′ terminus of the mRNA by a hairpin loop. This loop was then digested with the single-strand-specific nuclease S1 to give a double-stranded cDNA molecule that, after further enzymatic treatment, could be attached to a plasmid vector and propagated in *E. coli*. The self-primed synthesis of the second strand of cDNA was at best a poorly controlled reaction, and the subsequent cleavage of the hairpin structure almost invariably led to the loss or rearrangement of sequences corresponding to the 5′ terminus of the mRNA (Land et al. 1981). Consequently, this scheme for synthesizing double-stranded cDNA molecules has now been largely replaced by more efficient and less destructive methods (see below).

The conditions used to achieve synthesis of full-length second-strand cDNA depend on the particular polymerase used. Reactions employing *E. coli* DNA polymerase I are usually carried out at pH 6.9 to minimize the 5′→3′ exonuclease activity of the enzyme and at 15°C to minimize the possibility of synthesizing "snapback" DNA (see Chapter 10). These problems can be avoided completely by using either reverse transcriptase or the Klenow fragment of *E. coli* DNA polymerase I, both of which lack 5′→3′ exonuclease activity and are available in pure form from commercial sources.

Replacement Synthesis of the Second Strand of cDNA

In this method, which was introduced by Okayama and Berg (1982) and modified by Gubler and Hoffman (1983), the product of first-strand synthesis—a cDNA:mRNA hybrid—is used as a template for a nick-translation reaction (Figure 8.5). RNAase H produces nicks and gaps in the mRNA strand of the hybrid, creating a series of RNA primers that are used by *E. coli* DNA polymerase I during the synthesis of the second strand of cDNA. The reaction has three main virtues: (1) It is very efficient; (2) it can be carried out directly using the products of the first-strand reaction, which need no further treatment or purification; and (3) it eliminates the need to use nuclease S1 to cleave the single-stranded hairpin loop in the double-stranded cDNA—a reaction that is difficult to control and frequently results in a great loss of cDNA.

Most cDNA libraries are now constructed using a replacement reaction to synthesize the second strand of cDNA. The replacement reaction described by Gubler and Hoffman (1983) presents three theoretical problems. First, eukaryotic mRNAs carry "cap" structures at their 5′ termini, and it is not clear how these caps are removed so that the double-stranded cDNA can be inserted into a prokaryotic vector. Second, the replacement reaction is incapable of synthesizing the second strand of cDNA corresponding to the 5′-terminal region of the mRNA. Finally, hairpin priming of second-strand synthesis may still occur during the replacement reaction if RNAase H

efficiently removes the mRNA sequences immediately adjacent to the cap structure. In practice, however, these disadvantages turn out not to be serious: The products of the replacement reaction can be efficiently inserted into prokaryotic vectors without taking any special steps to remove the cap structure; the resulting cDNAs are often very nearly full-length, lacking only a few nucleotides corresponding to the 5′ terminus of the mRNA; and self-priming usually accounts for less than 10% of the second-strand synthesis.

Primed Synthesis of the Second Strand of cDNA

For most cloning purposes, the simple replacement reaction described by Gubler and Hoffman (1983) is perfectly adequate. However, if it is necessary to clone the 5′-terminal sequences of eukaryotic mRNA with high efficiency, several more-difficult methods are available. For example, after completion of first-strand synthesis, terminal transferase can be used to add homopolymeric tails of dC residues to free 3′-hydroxyl groups (Rougeon et al. 1975; Land et al. 1981). This tail is then hybridized to oligo(dG), which serves as a primer for synthesis of a full-length second strand of cDNA. The efficiency of this method seems to depend on the particular mRNA under study. In the best cases (e.g., chicken lysozyme mRNA [Land et al. 1981]), full-length clones of cDNA can be obtained with high efficiency; other mRNAs, however, have given more variable results (Rougeon et al. 1975; Cooke et al. 1980), perhaps reflecting the relative efficiencies with which

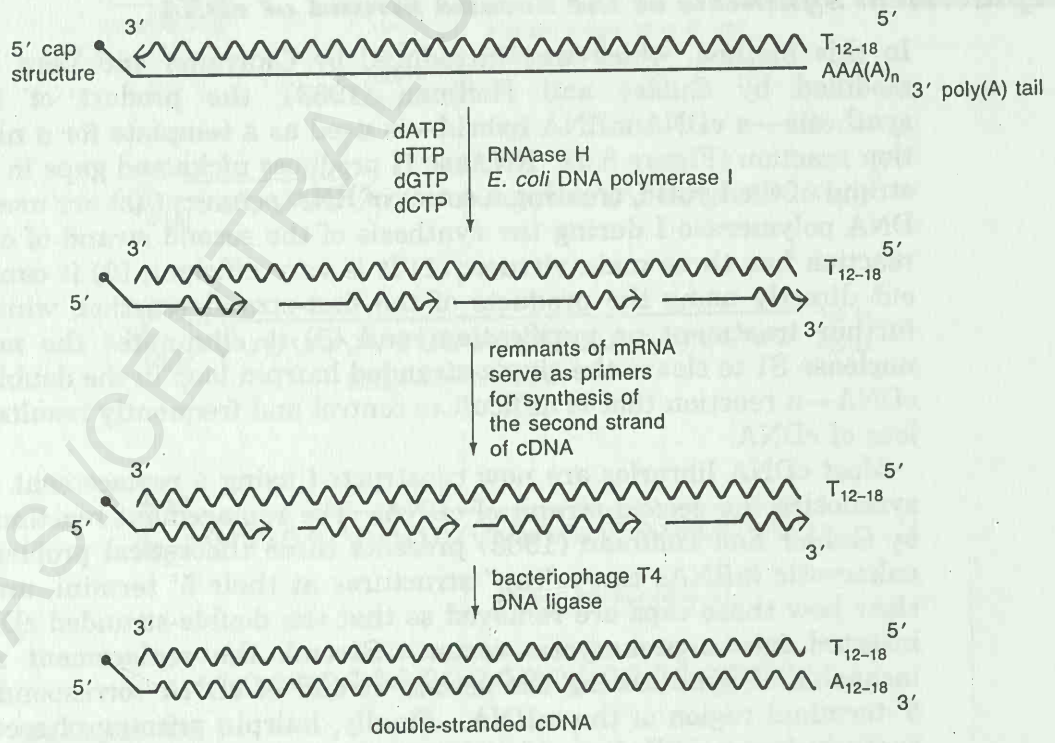

FIGURE 8.5
Replacement synthesis of double-stranded cDNA.

terminal transferase uses the 3′ termini of different first-strand cDNAs as substrates for homopolymeric tailing.

An important refinement to the process of second-strand synthesis was introduced by Okayama and Berg (1982), who used a fragment of plasmid DNA carrying a short tail of dG residues (Figure 8.6) as a primer. The dG residues are annealed to a homopolymeric tract of dC residues that has been added to the 3′ terminus of the first strand of cDNA by terminal transferase. Although the protocol generally works well, it is rather laborious, requiring the isolation of several different fragments of plasmid DNA, to which three separate sets of homopolymeric tails must be added. The process can be simplified by using synthetic primer-adapters that carry both homopolymeric tails for priming the synthesis of the first and second strands of cDNA and restriction sites for cloning into plasmids (Coleclough and Erlitz 1985) and bacteriophage λ vectors (Krawinkel and Zoebelein 1986; Han et al. 1987) (Figure 8.7). The use of primer-adapters has three advantages. First, the number of steps involved in the synthesis and cloning of cDNA is reduced. For example, in the protocol described by Han et al. (1987), the cDNA:mRNA hybrid is attached to a bacteriophage λ vector by synthetic adapters before synthesis of the second strand of cDNA. This eliminates several steps that are required when double-stranded cDNA is cloned by addition of synthetic linkers. Second, the increased efficiency of priming of second-strand synthesis yields libraries that contain a comparatively high proportion of full-length cDNA molecules. Third, the method can be adapted to allow the amplification of first-strand cDNA by the polymerase chain reaction (see Chapter 14). However, there are also some disadvantages. All of the clones generated by homopolymeric priming of second-strand synthesis carry a tract of dG:dC residues immediately upstream of the sequences corresponding to the sequences of the mRNA template. The presence of these additional sequences may inhibit transcription of DNA both in vivo and in vitro. Furthermore, they may form a barrier to the Klenow fragment of *E. coli* DNA polymerase I during DNA sequencing by the dideoxy-mediated chain-termination method, requiring that reverse transcriptase be used instead and the conditions adjusted accordingly.

Careful consideration should be given to the method used to synthesize the second strand of cDNA, since it can determine the choice of vector and can dictate the means used to link the cDNA to the vector. The available options are discussed in greater detail in the following sections.

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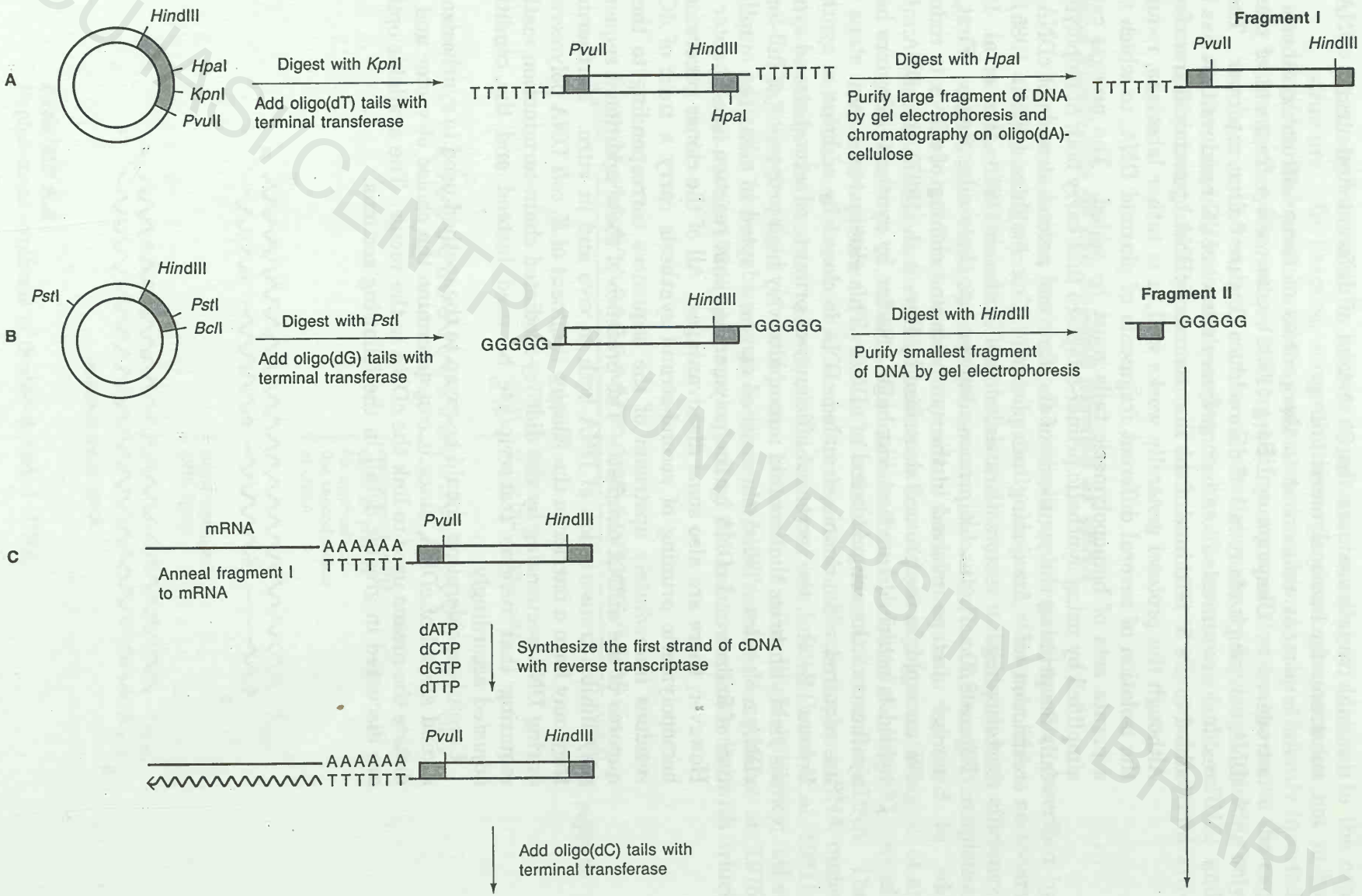

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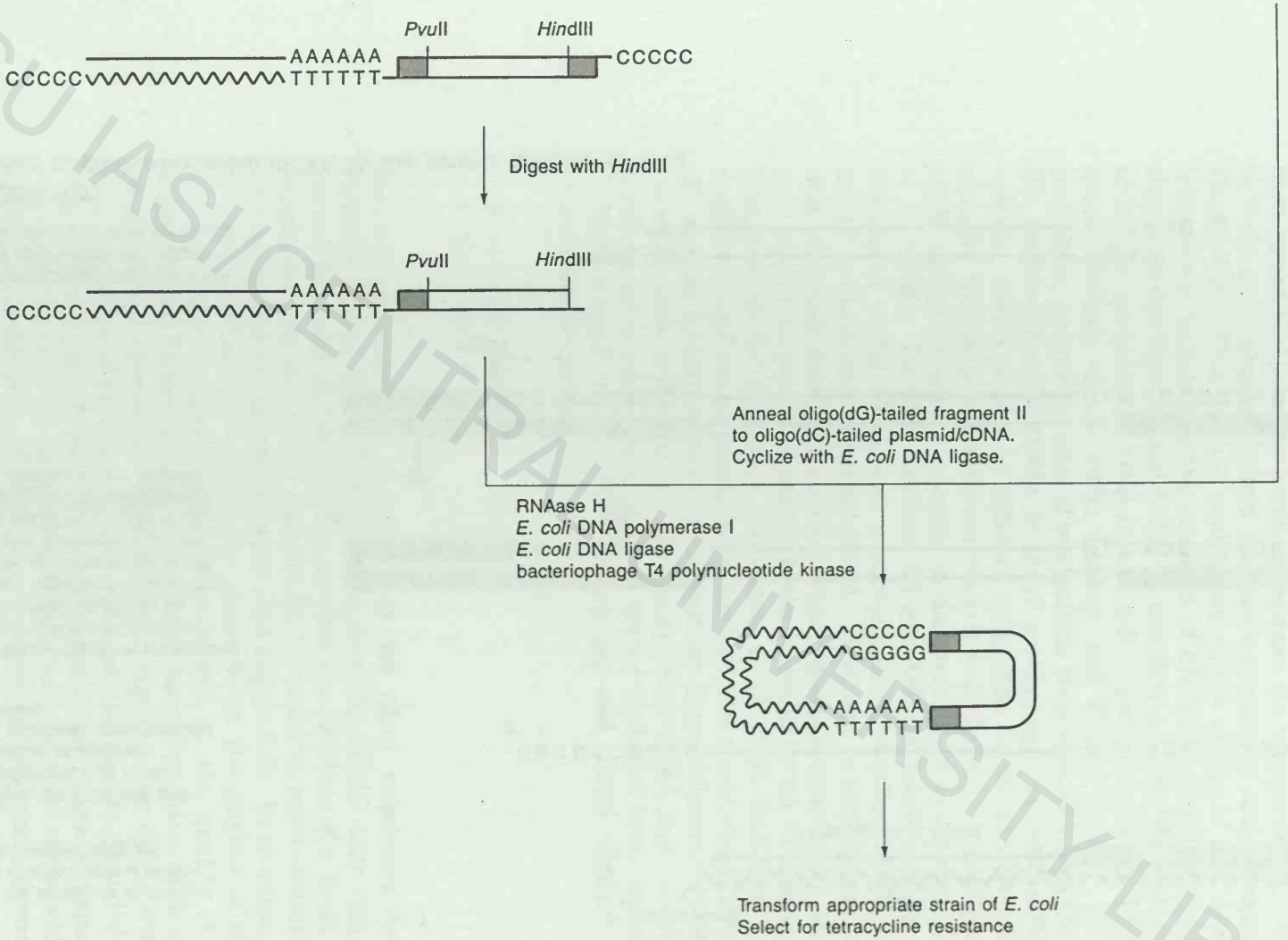

FIGURE 8.6
Cloning cDNA by the Okayama-Berg method.

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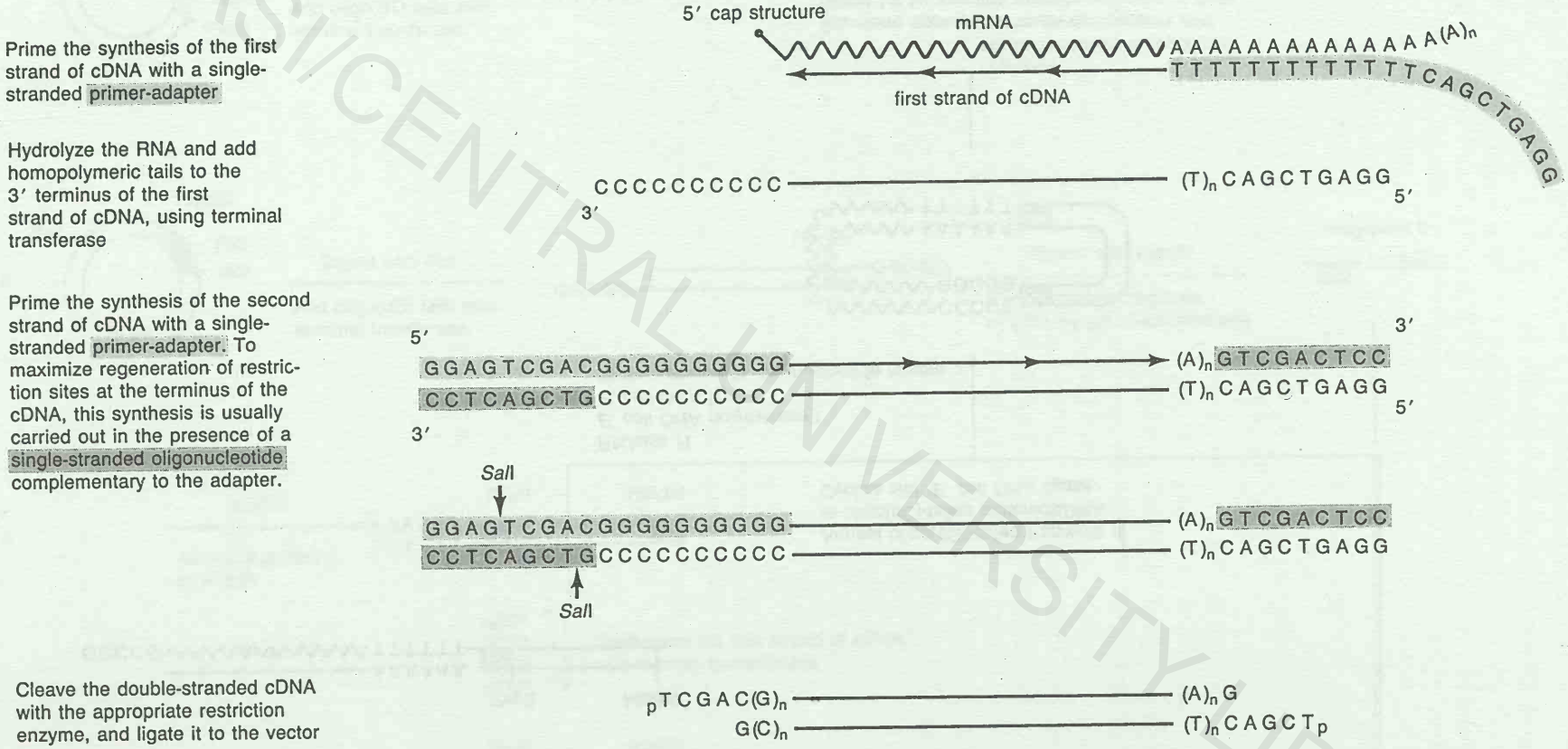

FIGURE 8.7
Synthesis of double-stranded cDNA by the primer-adapter method.

MOLECULAR CLONING OF DOUBLE-STRANDED cDNA

Homopolymeric Tailing

Until 1981, the most commonly used procedure for cloning cDNAs involved addition of complementary homopolymeric tracts to double-stranded cDNA and to the plasmid vector. The vector and the double-stranded cDNA were then joined by hydrogen bonding between the complementary homopolymers to form open circular hybrid molecules capable of transforming *E. coli* (see Figure 8.8). This method capitalized on the ability of calf thymus terminal transferase to catalyze the addition of dNTPs to the 3′-hydroxyl termini of single- or double-stranded DNA. It was first used by Wensink et al. (1974) to introduce recombinant DNA into *E. coli* by pairing between dA:dT homopolymers (Jackson et al. 1972; Lobban and Kaiser 1973). In the original procedure, a small number of nucleotides were removed from the 5′ termini of the double-stranded DNAs to leave protruding single-stranded 3′-hydroxyl termini, which served as efficient templates for terminal transferase. The need for this step was obviated when it was shown that terminal transferase could utilize recessed 3′ termini in the presence of cobalt ions (Roychoudhury et al. 1976). Usually, between 50 and 150 dA residues were added to the linearized vector DNA and a corresponding number of dT residues were added to the double-stranded cDNA. However, homopolymeric dA:dT tailing was used only rarely for cDNA cloning, chiefly because there was no satisfactory way to excise cDNA inserted into plasmids by dA:dT tailing; the methods described in the literature (Hofstetter et al. 1976; Goff and Berg 1978) are inefficient and not routinely reproducible. For many years, therefore, almost all cloning of cDNA was carried out by homopolymeric dC:dG tailing: dC residues were added to the double-stranded cDNA and complementary dG residues were added to a plasmid vector that had been digested with *Pst*I (Villa-Komaroff et al. 1978; Rowekamp and Firtel 1980). This enzyme cleaves the sequence

```
          ↓
5′ C T G C A G 3′
3′ G A C G T C 5′
     ↑
```

leaving protruding 3′ termini that are ideal substrates for addition of homopolymeric tails. cDNA clones carrying dC:dG tails can easily be removed from the plasmid vector because the addition of a short stretch of dG residues to the linearized plasmid DNA results in regeneration of a *Pst*I site at each end of the dC-tailed insert (see Figure 8.8). In practice, the efficiency of regeneration of the *Pst*I site depends on the quality of the restriction enzyme used to linearize the plasmid and on the quality of the terminal transferase. Contamination of either enzyme preparation with exonuclease or single-strand-specific nuclease prevents regeneration of the site.

A variation of this strategy is to insert the double-stranded cDNA into a site that is closely flanked by two hexanucleotide restriction sites. In pUC18, for example, the *Pst*I site is flanked by *Sal*I and *Sph*I sites, which occur only rarely in mammalian DNA. In most cases, cDNAs inserted into the *Pst*I site by homopolymeric tailing can be recovered intact by cleavage with *Sal*I and *Sph*I.

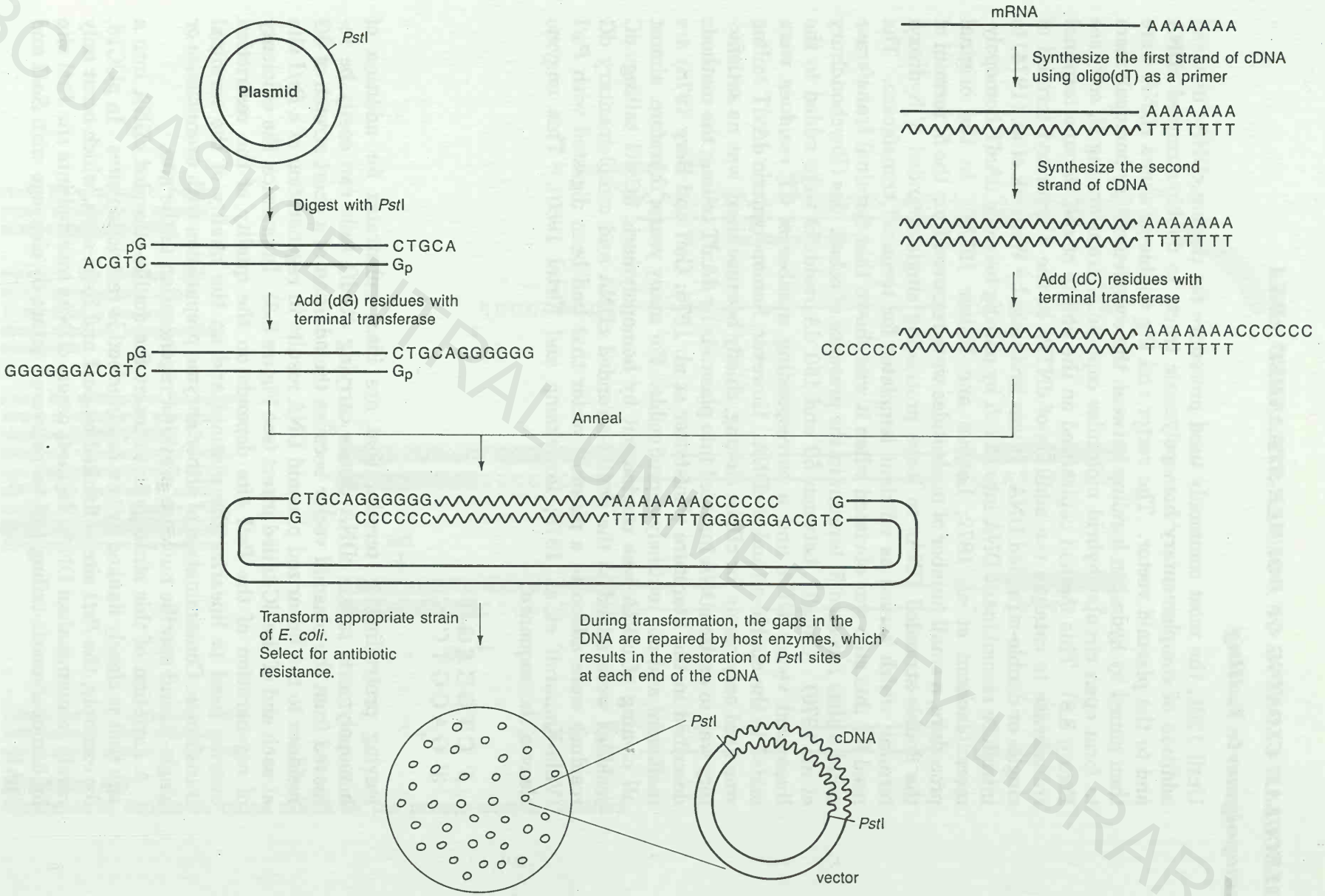

FIGURE 8.8
Cloning double-stranded cDNA by homopolymeric tailing.

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Although it was used with success for many years, cloning of homopolymerically tailed cDNAs into homopolymerically tailed vectors suffers from several drawbacks:

- The method can be used effectively only with plasmid vectors, which yield cDNA libraries that are much more difficult to store and replicate than those constructed in bacteriophage λ vectors.
- For optimal efficiency of cloning, the number of homopolymeric residues on the plasmid and the cDNA should be approximately equal, with approximately 100 dA/dT residues or 20 dC/dG residues being added to each end of the DNAs (Peacock et al. 1981). However, it is difficult to ensure that dNTPs are added at equal rates to the ends of all DNA molecules. The reaction often proceeds asynchronously, so that different termini of DNA molecules carry homopolymeric tails of very different lengths. This problem can be alleviated, but not completely solved, by using plasmids carrying homopolymeric tails of defined size, which are available from a number of commercial suppliers.
- Different strains of *E. coli* are transformed at different efficiencies with plasmid:cDNA molecules that are held together by complementary homopolymeric tails. For example, *E. coli* strain RR1, which is *recA*$^+$, yields ten times as many recombinant cDNA clones made by the dA:dT tailing procedure as the *recA*$^-$ strain HB101 yields (Peacock et al. 1981). Untreated pBR322 DNA transforms the two strains at equal efficiency. These results indicate that the bacterial *recA* system may be involved in repairing open circular hybrid DNA molecules that contain homopolymeric tails. Whether other bacterial genes are involved is unknown. However, the fact that the genetic background of the recipient strain of *E. coli* can markedly influence the efficiency of transformation by DNA molecules containing unligated homopolymeric tails raises the possibility of systematic selection for or against specific classes of plasmid:cDNA hybrids.

Synthetic DNA Linkers and Adapters

Synthetic linkers containing one or more restriction sites provide an alternative method for joining double-stranded cDNA to both plasmid and bacteriophage λ vectors. Double-stranded cDNA, generated by one of the methods described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I—enzymes that remove protruding single-stranded 3′ termini with their 3′→5′ exonucleolytic activities and fill in recessed 3′-hydroxyl termini with their polymerizing activities. The combination of these activities therefore generates blunt-ended cDNA molecules, which are then incubated with a very large molar excess of linker molecules in the presence of bacteriophage T4 DNA ligase, an enzyme that catalyzes the ligation of blunt-ended DNA molecules. The products of the reaction are cDNA molecules carrying polymeric linker sequences at their termini. These molecules are cleaved at a restriction site in the linker, purified, and ligated to a vector that has been cleaved with a restriction enzyme that generates cohesive termini compatible with those of the linker.

Double-stranded cDNA molecules containing the synthetic cohesive termini will of course ligate to each other as well as to the vector DNA. It is therefore important to arrange the ligation conditions to minimize the formation of these chimeric molecules, since it is extremely laborious to test subsequently whether cDNA clones that contain the particular restriction site at an internal location have been formed by end-to-end ligation of two unrelated cDNAs. The ligation mixture should therefore always contain a high molar excess of vector DNA to cDNA. However, these conditions strongly favor re-formation of the vector by self-ligation, leading to unacceptably high backgrounds of nonrecombinant clones. This problem may be minimized by treating the cleaved vector with a phosphatase before ligation to cDNA.

If the double-stranded cDNA contains one or more recognition sites for the restriction enzyme, it will be cleaved and subsequently cloned as two or more DNA fragments, making the isolation and analysis of full-length cDNAs difficult. Attempts to alleviate this problem have included:

- *The use of restriction enzymes that cleave mammalian DNA very rarely (e.g.,* Not*I and* Sal*I).* There is no guarantee that even a small cDNA does not contain a recognition site for a particular restriction enzyme. However, if the size of the cDNA clone of interest is considerably smaller than the size of the average fragment of genomic DNA obtained by digestion with the chosen enzyme, there is a good chance that it will not contain a recognition sequence for that enzyme. For example, the odds against finding the G/C-rich octanucleotide sequence recognized by *Not*I in a 5-kb clone of cDNA are less than 1 in 200. For *Sal*I, whose sites occur, on average, once every 100 kb in mammalian DNA, the odds rise to 1 in 20—still an acceptable risk, perhaps. In contrast, the average distance between *Eco*RI sites is approximately 4 kb, and so there is a reasonably good probability that a cDNA of average size will be cleaved into two or more pieces. If it is essential to clone into a restriction site that occurs as frequently as *Eco*RI, it is advisable to take one of the additional steps outlined below to avoid potential problems.
- *Treatment of double-stranded cDNA with a methylase (e.g., M.*Eco*RI methylase) before addition of synthetic linkers, so that naturally occurring restriction sites within the cDNA are protected from cleavage.* The linkers are then added to the termini of the cDNA and cleaved with the appropriate restriction enzyme to generate molecules with cohesive termini. These molecules are then purified and ligated to a large molar excess of the appropriate dephosphorylated vector.
- *The use of synthetic adapters rather than linkers.* Adapters are short, double-stranded oligonucleotides that carry one blunt end (which can be ligated to the double-stranded cDNA) and one cohesive terminus (which can be ligated to a compatible terminus in the vector). Unlike linkers, adapters do not require digestion with restriction enzymes after they have been ligated to double-stranded cDNA. However, cDNA molecules carrying phosphorylated adapters can form covalently closed circular molecules (which cannot be cloned) or chimeric linear molecules (which are highly undesirable) during the subsequent ligation reaction in the presence of

dephosphorylated vector DNA. A method to avoid this problem is shown in Figure 8.9. Only the shorter of the two oligonucleotides that make up the adapter is phosphorylated before ligation to cDNA. The products of the ligation (cDNA molecules carrying a single adapter at each end and dimer adapter molecules) are separated by chromatography through Sepharose CL-4B (see pages 8.70–8.72). The termini of the cDNA molecules carrying adapters are phosphorylated with bacteriophage T4 polynucleotide kinase and then ligated to a large molar excess of the appropriate dephosphorylated vector.

When joining linkers or adapters to blunt-ended double-stranded cDNA molecules, it is important that the ligation reaction be carried out in as small a volume as possible (to maintain as high a concentration as possible of linkers/adapters). The molar concentration of linkers/adapters must be at least 100 times greater than the concentration of termini of cDNA (to minimize blunt-end ligation of cDNA molecules). Finally, before the cDNA is inserted into the vector, unreacted adapters and the low-molecular-weight products created by restriction enzyme digestion of polymerized linkers must be efficiently removed by chromatography through columns (1-ml bed volume) of Sepharose CL-4B (see pages 8.70–8.72) or Bio-Gel A-50m (Huynh et al. 1985). Electrophoresis through agarose or polyacrylamide gels can also be used to separate unligated linkers/adapters from cDNA and to fractionate the cDNA by size. However, the recovery of cDNA from gels in a form that can be ligated efficiently to the vector can be poor, and column chromatography is therefore the method of choice for routine preparations of cDNA.

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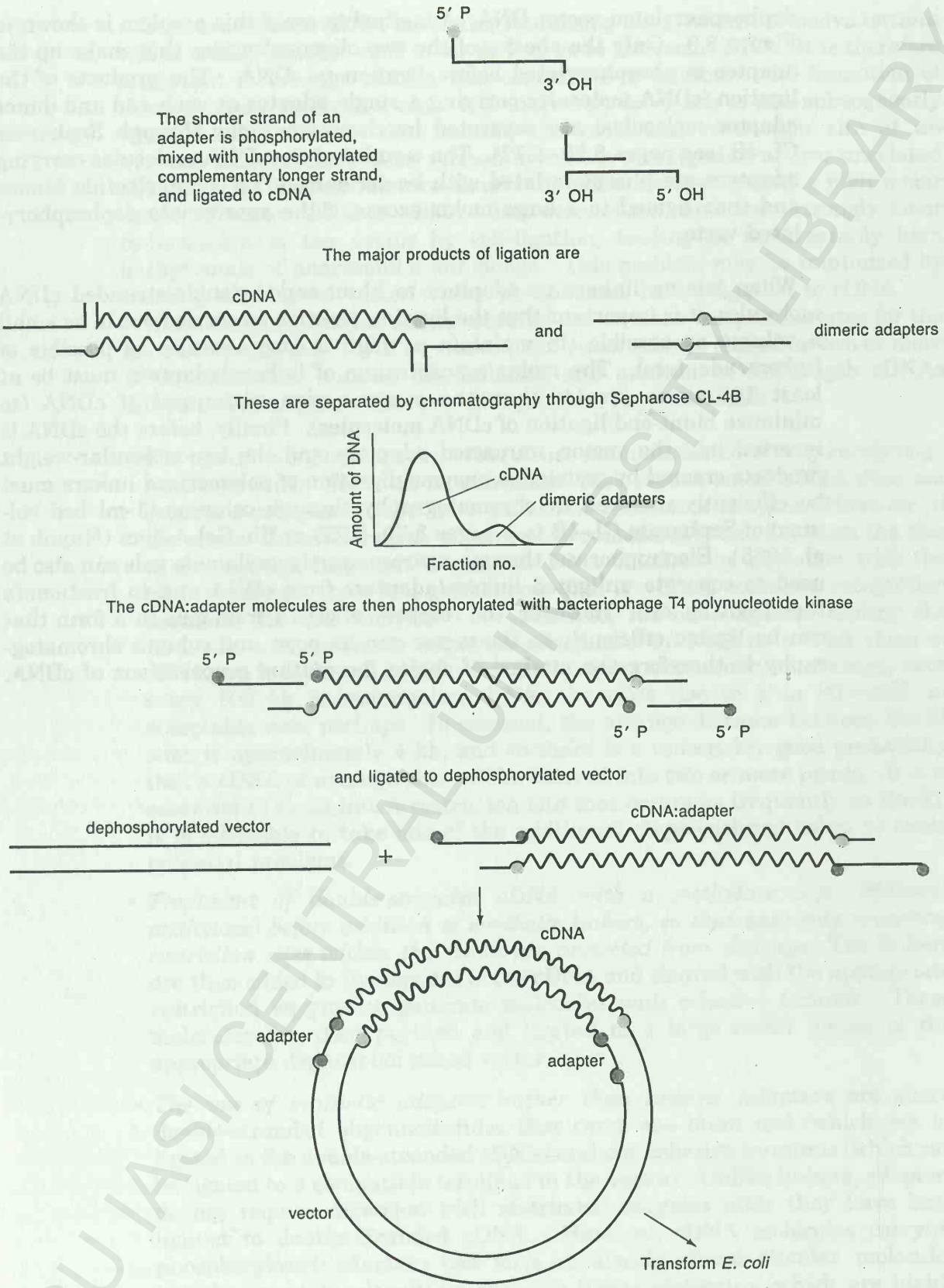

FIGURE 8.9
Cloning double-stranded cDNA using adapters.

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Alternative Methods of Cloning cDNA

mRNA-cDNA CLONING

Several groups have obtained cDNA clones by transformation of *E. coli* with mRNA:cDNA hybrids that have been joined to plasmid vectors (Wood and Lee 1976; Zain et al. 1979). The bacterial host removes the mRNA and replaces it with DNA. After the first strand of cDNA has been synthesized in the usual way, dA residues are added to the mRNA:cDNA hybrid, which is then annealed to a plasmid carrying dT tails. Because the efficiency of tailing the 3′-hydroxyl termini of RNA molecules is at least ten times less than that of the homologous reaction with DNA, most of the dA residues added to the hybrid are incorporated at the 3′-hydroxyl termini of the DNA strands. Joining of the other end of the hybrid to the vector is probably accomplished by hydrogen bonding between the tract of natural poly(A) at the 3′ terminus of the mRNA and the dT-tailed plasmid. The chief practical advantage of this procedure is that no synthesis of the second strand of cDNA is required. Furthermore, the procedure should, in theory, allow the sequences at the 5′ terminus of the mRNA (which are often lost during conventional cloning procedures) to be cloned. Its major disadvantage, however, is that it is at least ten times less efficient than double-stranded cDNA cloning and is therefore unsuitable for constructing large numbers of cDNA clones.

SEQUENTIAL ADDITION OF DIFFERENT LINKERS

In this method (Helfman et al. 1983) (Figure 8.10), double-stranded cDNA synthesized by the self-priming mechanism is ligated to one type of synthetic linker before the hairpin loop in the cDNA is cleaved. The linkers are therefore added only to one end of the double-stranded cDNA (the end corresponding to the 3′ terminus of the mRNA). The cDNA is then purified from excess unligated linkers, and the hairpin loop is cleaved with nuclease S1 and repaired with the Klenow fragment of *E. coli* DNA polymerase I before linkers of a second type are ligated to the cDNA. These linkers will be added to both ends of the cDNA. The double-linkered cDNA is then treated with the appropriate restriction enzymes and inserted into a vector by directional cloning.

This method has been used to insert cDNA in the correct orientation into plasmid vectors that allow expression of the inserted sequences in bacteria (see Chapter 1). Libraries established in this way may be screened for the presence of material that reacts with antisera specific for a particular gene product. Bacteriophage λ vectors that allow for directional cloning have become available only recently.

When this method was first described (Kurtz and Nicodemus 1981; Helfman et al. 1983), it suffered from a major drawback—the double-stranded cDNA needed to be digested with the appropriate restriction enzymes to generate cohesive termini. If the cDNA contained one or more recognition sites for either of the enzymes, it would be cleaved and cloned as two or more DNA fragments, making the structural analysis of the full-length cDNA difficult. This problem can now be alleviated by treating the double-stranded cDNA with both of the appropriate bacterial methylases before addition of

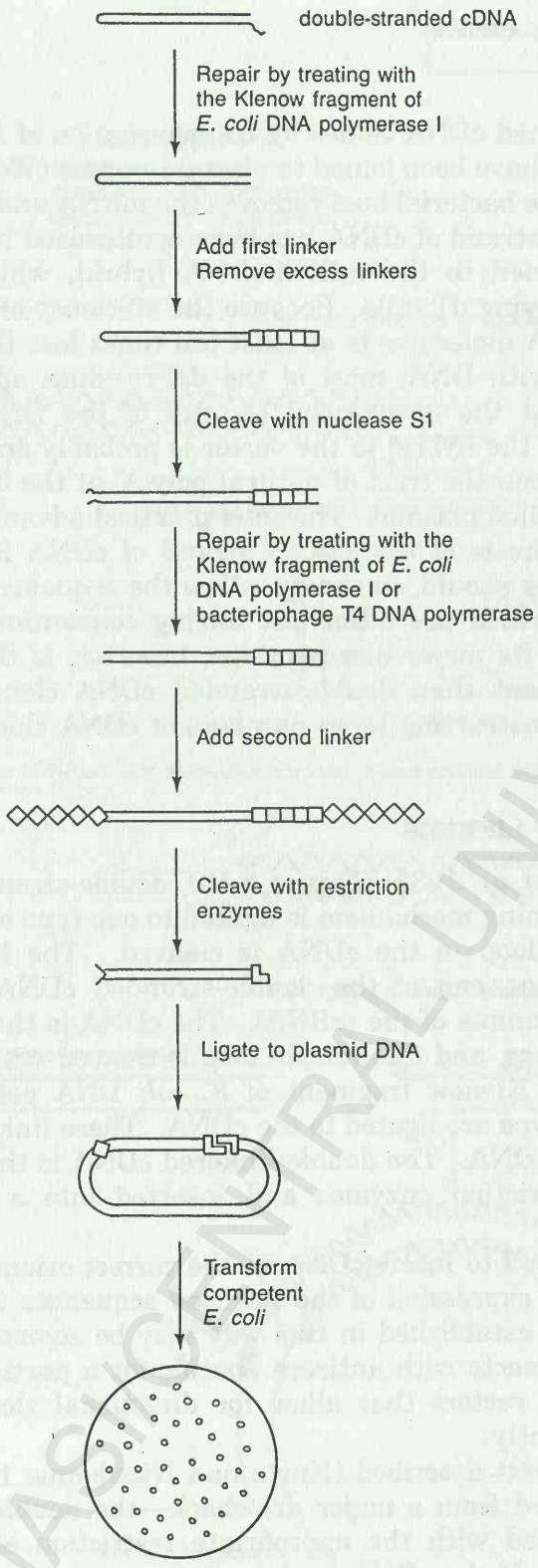
double-stranded cDNA
Repair by treating with the Klenow fragment of E. coli DNA polymerase I
Add first linker
Remove excess linkers
Cleave with nuclease S1
Repair by treating with the Klenow fragment of E. coli DNA polymerase I or bacteriophage T4 DNA polymerase
Add second linker
Cleave with restriction enzymes
Ligate to plasmid DNA
Transform competent E. coli

FIGURE 8.10
Cloning cDNA by sequential addition of synthetic DNA linkers.

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the first set of linkers, by using adapters instead of linkers, or by using linkers that contain restriction sites rarely found in mammalian cells (e.g., *Sal*I or *Not*I). However, some difficulties still remain. First, the method depends on the ability of the first strand of cDNA to serve as a primer-template for the synthesis of the second strand. This reaction is far less efficient than replacement synthesis of the second strand. Second, the method generates cDNA libraries in plasmid vectors, which are more difficult to maintain and screen than those constructed in bacteriophage λ vectors. For these reasons, immunological screening of cDNA libraries is usually carried out with libraries constructed in the bacteriophage λ expression vectors λgt11 and its derivatives, λORF8, and λZAP.

cDNA CLONING IN OKAYAMA-BERG VECTORS

As mentioned earlier, a novel method for high-efficiency cloning of full-length double-stranded cDNA was developed by Okayama and Berg (1982, 1983). Synthesis of the first strand of cDNA is primed by oligo(dT) covalently joined to a linear plasmid DNA. Homopolymeric dC tails are then added to the 3′-hydroxyl terminus of the newly synthesized cDNA, and the dC tails added simultaneously to the 3′ terminus of the plasmid are removed by digestion with a restriction enzyme. The plasmid vector is then circularized with a linker DNA that carries a cohesive terminus at one end and a homopolymeric tail of dG residues at the other. The dG residues pair with the dC tail of the first strand of cDNA and serve as primers for replacement synthesis of the second strand of cDNA (see Figure 8.6). This scheme was modified subsequently so that it could be used with bacteriophage λ vectors (Okayama and Berg 1985).

When it was first described, this method represented a considerable advance over the schemes for synthesis and cloning of cDNA that were then in common use. Chief among its advantages was the use of a very efficient replacement reaction mediated by RNAase H and *E. coli* DNA polymerase I for the synthesis of the second strand of cDNA. In addition, the cDNA was covalently attached to the vector during synthesis, thereby eliminating such problems as removal of excess linkers from the cDNA preparation. Finally, the cDNA could be synthesized in a vector that would allow expression in mammalian cells, opening the possibility of screening transfected cells directly for functional proteins (Okayama and Berg 1985; Wong et al. 1985; Yang et al. 1986). However, the method also suffers from several impediments:

- It depends on the addition of three sets of homopolymeric tails to separate termini of DNA. Tailing reactions with terminal transferase are difficult to both monitor and control, and it is often necessary to repeat each reaction several times before a satisfactory result is obtained. This problem can be partially eliminated by using Okayama-Berg vectors carrying homopolymeric tails prepared by commercial manufacturers. However, the reaction in which the first strand of cDNA is tailed with dC—the most difficult of the three reactions to control—must still be carried out in the laboratory.

- The synthesis of the first strand of cDNA is comparatively inefficient, since it is often difficult to incorporate sufficient dT-tailed vector in the reaction to achieve an optimal ratio of primer to template.

- The final cDNA clone is bounded on both sides by complementary homopolymers (dA:dT at the 3′ terminus of the coding strand and dC:dG at the 5′ terminus). The presence of these homopolymeric tracts is a disadvantage, since they may inhibit expression of the cloned cDNA in mammalian cells and almost certainly will act as a barrier to the Klenow fragment of *E. coli* DNA polymerase I, so that sequencing by the dideoxy chain-termination method cannot be performed easily with this enzyme. Furthermore, the cDNA sequences cannot be excised from the vector without these flanking tails, which can only be removed from individual clones with great difficulty.

Despite these problems, the Okayama-Berg scheme was of great importance, since it pioneered the development of the highly efficient replacement reaction for synthesis of the second strand of cDNA. This reaction forms the basis of many of the protocols for cDNA cloning that are in current use.

cDNA CLONING WITH PRIMER-ADAPTERS

The ready availability of oligonucleotides of defined sequence has led to the development of cloning methods employing primer-adapters that contain a tract of homopolymeric DNA at the 3′ terminus and a restriction site at the 5′ terminus (Coleclough and Erlitz 1985; Krawinkel and Zoebelein 1986; Han et al. 1987). The homopolymeric sequences are used to prime the synthesis of the first or second strand of cDNA, and the flanking restriction sites are used to insert the final double-stranded cDNA molecules into an appropriate vector. In its simplest form, this method allows cDNAs to be cloned with high efficiency, without regard to orientation within the vector (see Figure 8.7). cDNAs inserted in this way into expression vectors such as λgt20 and λgt22 have only one chance in six of being expressed in bacteria: Only one half of the cDNA molecules will be inserted into the vector in the correct orientation with respect to the *lacZ* promoter, and only one in three of the molecules inserted in the right orientation will be in the correct reading frame to generate a fusion protein.

More complicated schemes employing primer-adapters can be devised to ensure that the 5′ terminus of the coding strand of cDNA is oriented immediately downstream from the promoter. Because the second strand of cDNA is synthesized after the first strand of cDNA has been attached to the vector, these schemes also eliminate the need to cleave the double-stranded cDNA with a restriction enzyme. For example, Coleclough and Erlitz (1985) used a primer-adapter (5′ GATCCACGCGT$_{15}$) to prime synthesis of the first strand of cDNA. After removal of the RNA template and elimination of unincorporated primer by alkaline agarose gel electrophoresis, homopolymeric dC tails were added to the 3′ terminus of the single-stranded cDNA. The 5′ termini of the resulting molecules were then ligated to linear plasmid DNA carrying a *Bam*HI site at one end and a homopolymeric stretch of dG residues at the other. The efficiency of ligation of the 5′ terminus of the

single-stranded cDNA to the vector is normally very poor, but it can be improved dramatically by including a molar excess of single-stranded "partners" in the reaction. These partners (5′ A_8CGCTC) bind to complementary sequences of the primer-adapter, forming small double-stranded segments that can establish phosphodiester bonds with both strands of the bacteriophage λ vector. After attachment of the cDNA to the vector, the second strand of cDNA is synthesized by a conventional extension reaction using the homopolymeric tract of dG residues as a primer.

In a similar protocol, Han et al. (1987) use a synthetic primer-adapter consisting of a *Sal*I site and a homopolymeric tract (5′ GAATTCGTCGACT_{15}) to prime the synthesis of the first strand of cDNA. After fractionation of the cDNA by alkaline agarose gel electrophoresis, approximately 15 dG residues are added to the 3′ terminus of the cDNA and a second primer-adapter (5′ GAATTCGTCGACC_{15}) is then annealed to it. Because their protruding 3′ termini are different from one another, the resulting double-stranded cDNA molecules can be efficiently ligated, in the presence of suitable partners, in only one orientation to bacteriophage λ arms that have been prepared by cleavage of λgt20–23 with *Sal*I and *Xba*I. After attachment of the cDNA to the vector, the second strand of cDNA is synthesized by extension of the homopolymeric tract of dC residues.

cDNA cloning with primer-adapters offers several advantages. First, it guarantees the production of recombinant genomes that carry their cDNAs in the correct orientation for expression. The number of recombinants to be screened can therefore be reduced by a factor of 2. This can be important when antiserum is in short supply or when the mRNA of interest is extremely rare, for example, when the gene of interest is expressed at low levels in only a subpopulation of the cells used as the source of mRNA. Second, the cDNA is spared from digestion with restriction enzymes. Third, because they require fewer enzymatic reactions, these methods offer the prospect of improving the efficiency with which small amounts of mRNA or cDNA (e.g., those obtained by subtractive hybridization, see Chapter 10) might be cloned. Despite these appealing characteristics, synthesis and cloning of cDNA with primer-adapters has not yet found wide acceptance. The current protocols require the elimination of small oligonucleotides and unused primers from the first strand of cDNA by electrophoresis through alkaline agarose gels. The recovery of single-stranded cDNA from such gels is usually poor, and special efforts are often required (e.g., reversed-phase chromatography) to eliminate contaminants that inhibit terminal transferase. The subsequent addition of homopolymeric tails to the 3′ terminus of the cDNA is often difficult to control and monitor. During these steps, it is essential that the integrity of the 5′ terminus of the cDNA be maintained. Unfortunately, many batches of commercially available terminal transferase and reverse transcriptase contain a 5′ exonucleolytic activity that can destroy the ability of the primer-adapter sequences at the 5′ terminus of the cDNA to anneal to complementary sequences in the vector. The role of complementary oligonucleotides in the ligation reaction has not yet been analyzed in detail, and the relative efficiency of the reaction is therefore unknown. Finally, because the range of primer-adapters available from commercial sources is quite limited, it may also be necessary to synthesize, purify, and test them before constructing the cDNA library.

Although there may be special circumstances that call for the use of primer-adapters or other experimental procedures, we recommend that routine synthesis and cloning of cDNA be carried out by oligo(dT)-priming of the first strand, followed by replacement synthesis of the second. For construction of libraries that are to be screened only by nucleic acid hybridization, cloning in λgt10 using *Eco*RI linkers is the best choice. If there is a possibility that the library may also be screened by expression, the use of *Not*I linkers in a vector such as λZAP or λgt22 is preferred. However, it may be prudent also to construct a library in λZAP or λgt22 by addition of *Eco*RI linkers after protection of internal *Eco*RI sites by methylation.

cDNA CLONING IN SINGLE-STRANDED VECTORS

This method of cloning is used to generate cDNA libraries that can be conveniently screened by hybrid depletion. Unlike hybrid selection, which can only be used to screen for clones corresponding to individual subunits of proteins, hybrid depletion can identify cDNA clones encoding any subunit of a multisubunit protein (Lübbert et al. 1987). The principle of the method is shown in Figure 8.11. A population of mRNA molecules that includes species encoding all of the subunits of a multifunctional protein is hybridized to pools of cDNA clones constructed in a single-stranded vector. If the mRNA encoding one of the subunits of the protein forms a DNA:RNA hybrid, a depleted population of mRNA molecules is obtained that is no longer fully capable of directing the synthesis of the functional protein. Hybrid depletion can therefore be used to identify cDNA clones encoding essential polypeptide subunits for which no immunological or nucleic acid reagents are available. However, the method can be used only when a sensitive functional assay for the complete protein is available.

Single-stranded vectors are preferred because (1) they allow the use of liquid-phase hybridization reactions, which are more efficient than reactions involving immobilized nucleic acids, and (2) they allow DNA:RNA hybrids to be removed by equilibrium centrifugation in CsCl gradients containing guanidine HCl (Enea and Zinder 1975).

Hybrid depletion requires that the single-stranded recombinants contain cDNAs in the anti-sense orientation so that these inserts can be hybridized to their corresponding mRNAs without complications caused by DNA:DNA annealing. The directional cloning scheme devised by Lübbert et al. (1987) (Figure 8.12) uses the phagemid pUC119 as a vector. As described in Chapter 3, bacteria carrying recombinant phagemids that are superinfected with an appropriate helper bacteriophage produce virus particles whose genomes contain single-stranded copies of the cloned DNA sequence. This cloning scheme has two other advantages. It allows single-stranded DNA of the desired size to be cloned and, by the use of primer-adapters, it avoids digesting double-stranded cDNA with restriction enzymes. λZAP is particularly well-suited for this type of cloning, since cDNA libraries constructed in this vector can easily be converted to phagemid libraries in vivo, using superinfection with f1 helper bacteriophages.

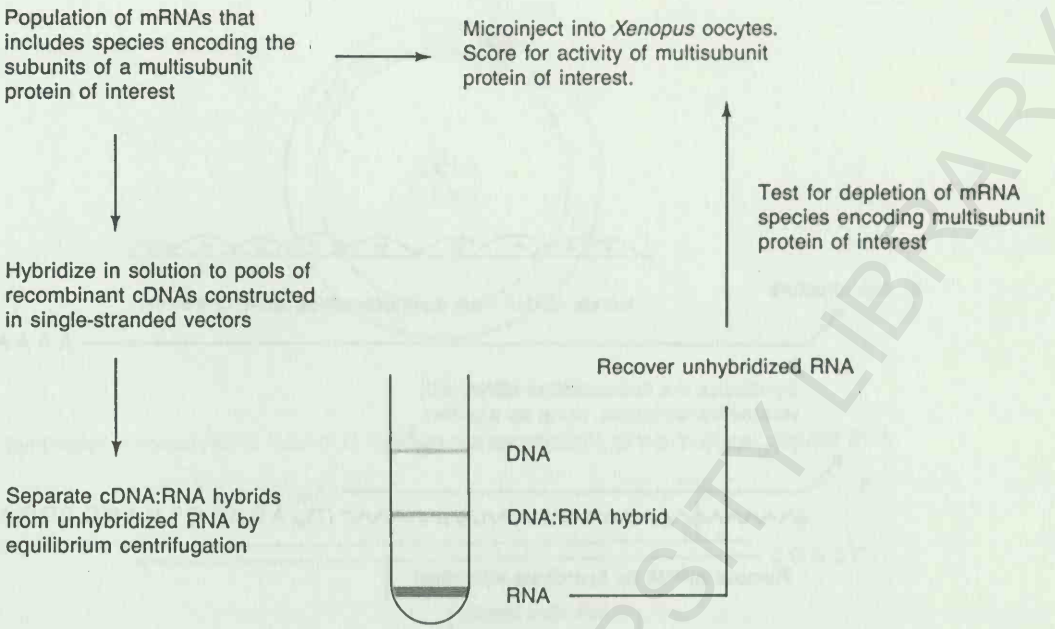
Population of mRNAs that includes species encoding the subunits of a multisubunit protein of interest
Microinject into *Xenopus* oocytes. Score for activity of multisubunit protein of interest.
Test for depletion of mRNA species encoding multisubunit protein of interest
Hybridize in solution to pools of recombinant cDNAs constructed in single-stranded vectors
Recover unhybridized RNA
DNA
DNA:RNA hybrid
RNA
Separate cDNA:RNA hybrids from unhybridized RNA by equilibrium centrifugation

FIGURE 8.11
Screening cDNA clones by hybrid depletion.

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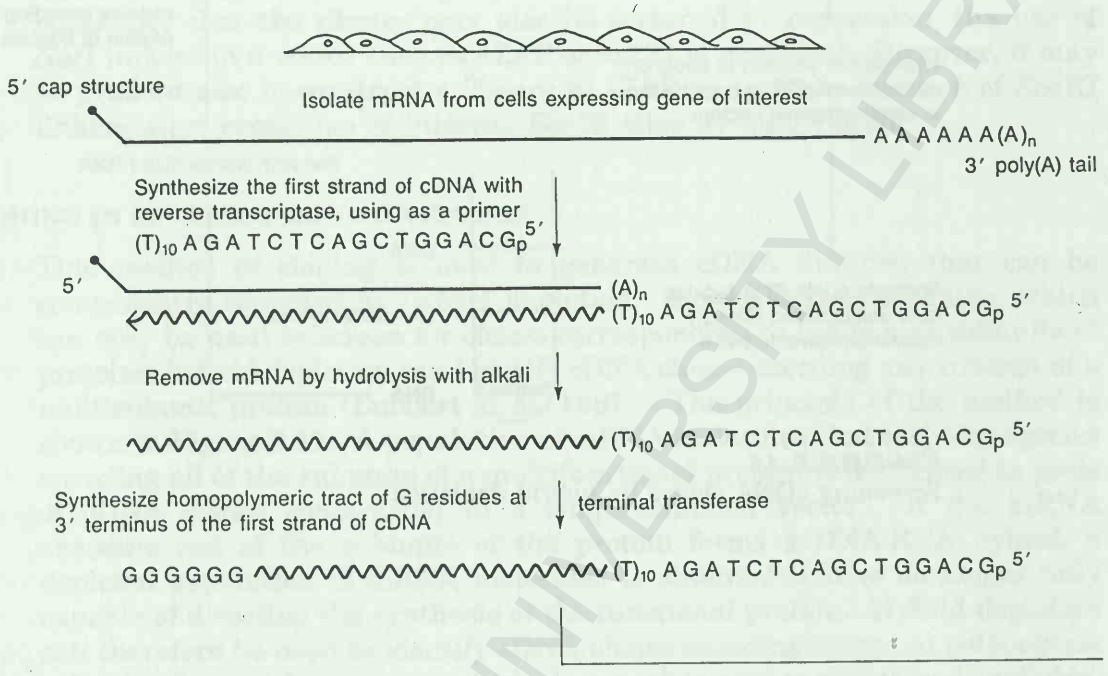
5′ cap structure
Isolate mRNA from cells expressing gene of interest
A A A A A A $(A)_n$
3′ poly(A) tail
Synthesize the first strand of cDNA with reverse transcriptase, using as a primer
$(T)_{10}$ A G A T C T C A G C T G G A C G_p 5′
5′
$(A)_n$
$(T)_{10}$ A G A T C T C A G C T G G A C G_p 5′
Remove mRNA by hydrolysis with alkali
$(T)_{10}$ A G A T C T C A G C T G G A C G_p 5′
Synthesize homopolymeric tract of G residues at 3′ terminus of the first strand of cDNA
terminal transferase
G G G G G G
$(T)_{10}$ A G A T C T C A G C T G G A C G_p 5′

FIGURE 8.12
Cloning double-stranded cDNA by homopolymer priming.

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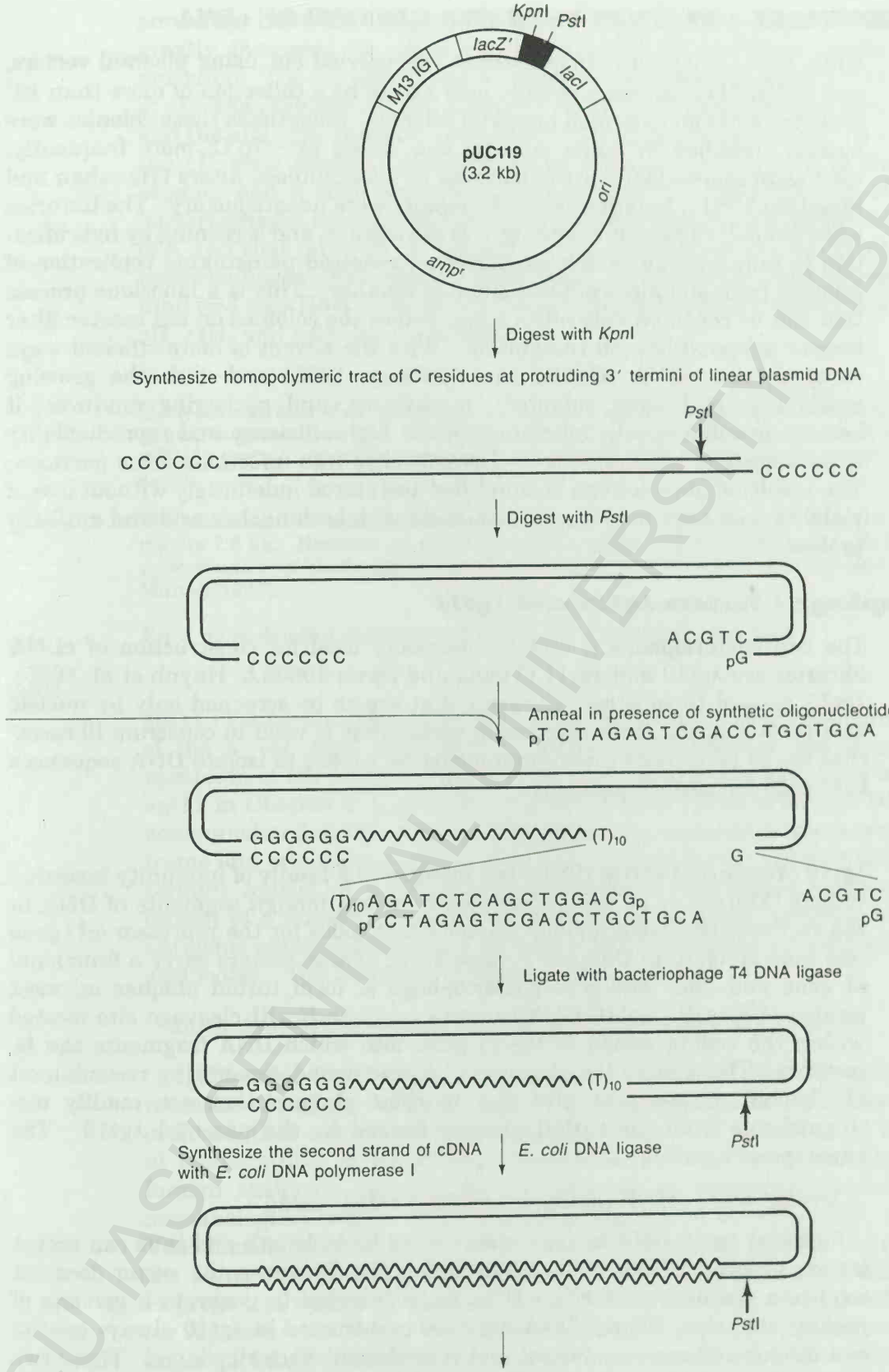

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BACTERIOPHAGE λ VECTORS USED FOR CLONING OF cDNA

Until 1983, almost all cDNA cloning was carried out using plasmid vectors, and cDNA libraries were usually maintained as a collection of more than 10^5 independently transformed bacterial colonies. Sometimes these colonies were pooled, amplified in liquid culture, and stored at −70°C; more frequently, they were maintained on the surfaces of nitrocellulose filters (Hanahan and Meselson 1983). In either case, the results were unsatisfactory. The libraries were difficult to preserve without loss of viability, and screening by hybridization to multiple radioactive probes often required painstaking replication of colonies from one nitrocellulose filter to another. This is a laborious process that can be repeated only a few times before the colonies on the master filter become smeared beyond recognition. With the advent of more efficient ways to synthesize cDNA (Gubler and Hoffman 1983) and with the growing availability of linkers, adapters, methylases, and packaging mixtures, it became possible to take advantage of the high efficiency and reproducibility of packaging of bacteriophage λ DNA in vitro into infectious virus particles. The resulting libraries can be amplified and stored indefinitely without loss of viability, and they can be screened easily with both nucleic acid and antibody probes.

Bacteriophage λ Vectors λgt10 and λgt11

The two bacteriophage λ vectors commonly used for construction of cDNA libraries are λgt10 and λgt11 (Young and Davis 1983a,b; Huynh et al. 1985). λgt10 is used to construct libraries that are to be screened only by nucleic acid probes; λgt11 is an expression vector that is used to construct libraries that are to be screened with immunological probes to isolate DNA sequences that code for specific antigens.

λgt10

λgt10 (Young and Davis 1983a) is a member of a family of immunity insertion vectors (Murray et al. 1977) designed to accept foreign segments of DNA in the region of the bacteriophage genome that codes for the repressor (cI) gene (see map of λgt10 in Chapter 2, page 2.40). These vectors carry a functional *c*I gene and, like wild-type bacteriophage λ, form turbid plaques on most strains of *E. coli*. λgt10 DNA contains a single *Eco*RI cleavage site located within the coding region of the *c*I gene into which DNA fragments can be inserted. This causes the *c*I gene to be inactivated, generating recombinant $c\mathrm{I}^-$ bacteriophages that give rise to clear plaques that are readily distinguishable from the turbid plaques formed by the parental λgt10. The genotype of λgt10 is:

$$\lambda sr\mathrm{I}\lambda 1^\circ\ b527\ sr\mathrm{I}\lambda 3^\circ\ imm434\ (sr\mathrm{I}434^+)\ sr\mathrm{I}\lambda 4^\circ\ sr\mathrm{I}\lambda 5^\circ$$

Parental λgt10 DNA is approximately 43 kb in length and thus can accept inserts of foreign DNA up to 7.6 kb in length. However, the vector does not require a fragment of foreign DNA to be inserted to generate a genome of packageable size. Thus, cDNA libraries constructed in λgt10 always consist of a mixture of nonrecombinant and recombinant bacteriophages. These two types of bacteriophages can be distinguished phenotypically by their ability to form clear (recombinant) or turbid (nonrecombinant) plaques. More im-

portantly, however, the proportion of recombinants in these libraries can be greatly increased by plating on a strain of *E. coli* that carries an *hfl*$^-$ mutation. The *c*II gene of bacteriophage λ is a positive activator of synthesis of *c*I. The stability of the *c*II gene product is controlled by the host *hflA* gene and the *c*III gene product. Mutation of the *hflA* gene enhances the stability of the *c*II gene product, resulting in an increased production of the *c*I gene product and a high frequency of lysogenization. The growth of parental bacteriophages that carry an intact *c*I gene is therefore suppressed on strains of *E. coli* that carry an *hfl*$^-$ mutation. In contrast, the growth of recombinant bacteriophages that cannot synthesize an active *c*I gene product is not inhibited (see Hoyt et al. [1982] for a discussion of the interactions of the *c*I, *c*II, *c*III, and *hflA* gene products).

Notes

Many *hfl*$^-$ strains of *E. coli* are *recA*$^+$. In such hosts, recombination can in theory result in rearrangement or loss of cDNA sequences. Fortunately, only a small minority of mRNAs contain repetitive sequences and recombination between them seems to be a rare event.

The maximum theoretical size of the cDNA that can be propagated in λgt10 is approximately 7.6 kb. However, to allow a margin of safety, cDNA estimated to be larger than approximately 6 kb should be cloned in a vector with a greater capacity (see Kaiser and Murray 1985).

For a more detailed description of λgt10, see Chapter 2, page 2.41.

λgt11

λgt11 (Young and Davis 1983a,b) is an expression vector that carries a copy of the *E. coli lacZ* gene, with a single *Eco*RI cleavage site located 53 bp upstream of the translational termination codon of the *lacZ* gene (see map of λgt11 in Chapter 2, page 2.42). Approximately 7.2 kb of foreign DNA can be accommodated at this site. Coding sequences inserted in the correct reading frame and orientation will be expressed to yield fusion proteins whose amino termini consist of β-galactosidase sequences and whose carboxyl termini consist of a foreign polypeptide. Some of these fusion proteins will display antigenic epitopes that can be detected by their ability to react with specific antibodies. The genotype of λgt11 is:

λ*lac*5 Δ*shn*dIIIλ2-3 *sr*Iλ3° c*Its*857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

λgt11 codes for a temperature-sensitive repressor (c*Its*857) and an amber mutation (*S*am100) in the lysis gene. It can therefore form plaques on strains of *E. coli* that carry the amber suppressor *supF* and can give rise to lysogens at temperatures at which the repressor is active. Fusion proteins carrying foreign antigenic epitopes can be detected by screening plaques with an immunological probe. Bacteriophages are plated at 42°C on *E. coli* strain Y1090*hsdR* (relevant genotype: *hsdR supF* Δ*lac* Δ*lon* pMC9). After 4 hours, the plates are shifted to 37°C and overlaid with a sterile nitrocellulose filter impregnated with isopropylthio-β-D-galactoside (IPTG), which inactivates the *lac* repressor and induces expression of the fusion protein. After several more hours of incubation at 37°C, the filters are incubated with antibodies that bind to the antigen of interest. These antibodies are then detected by histochemical or radiochemical tests (see Chapter 12).

Notes

Until recently, cDNA libraries constructed in λgt11 were usually propagated on *E. coli* strain Y1088 (relevant genotype: *supE supF hsdR Δlac* pMC9). The *lac* deletion prevents recombination between the host and bacteriophage genomes; *supF* suppresses the amber mutation in the lysis (*S*) gene of the bacteriophage and allows λgt11 to lyse the infected cells; plasmid pMC9 expresses high levels of the *lac* repressor (the product of the *lacI* gene), which prevents expression of the fusion protein during the initial stages of plaque formation. Thus, synthesis of potentially toxic products is arrested until the infecting bacteriophage has completed several rounds of growth. Most importantly, Y1088 is defective for host-controlled restriction of DNA (*hsdR*4$^-$), but it carries an active modification system. Recombinant bacteriophages propagated in strain Y1088 are therefore protected from restriction when they are used to express foreign proteins in a restriction-competent, protease-deficient host such as *E. coli* strain Y1090. Unfortunately, for reasons that are not known, λgt11 forms very small plaques in strain Y1088, and so it is often difficult to measure an accurate titer of a bacteriophage stock on this host. Recently, this problem was circumvented by introducing the *hsdR*4$^-$ mutation into strain Y1090 (Jendrisak et al. 1987). λgt11 forms large plaques on this host and yields plate stocks of high titer. Strains of *E. coli* Y1090 carrying the *hsdR*4$^-$ mutation are available from Promega and other suppliers.

λgt11 expresses an active repressor (cI) protein and can form lysogens efficiently at 32°C. In the original description of this vector (Young and Davis 1983a), libraries of bacterial lysogens carrying recombinant λgt11 genomes were induced by heating and screened for the subsequent production of foreign antigens. Propagation of recombinant bacteriophages as lysogens appeared to offer certain advantages: λgt11 genomes carried and inherited as part of the host-cell chromosome would perhaps be more stable than genomes propagated by bacteriophage infection; furthermore, the repression of synthesis of potentially toxic fusion proteins would be more complete. Despite these apparent advantages, libraries constructed in λgt11 are no longer propagated and screened as bacterial lysogens because it was found that some λgt11 recombinants are unable to form stable lysogens (Huynh et al. 1985). This problem could not be solved by the use of *hflA* strains of *E. coli*, which have increased efficiency of lysogenization, nor by the incorporation of a selectable marker (*amp*^{r}) into the λgt11 genome. λgt11 libraries are now propagated by lytic growth, and plaques, rather than induced lysogenic colonies, are screened with immunological probes (Young and Davis 1983b).

Libraries constructed in λgt11, like those constructed in λgt10, can be screened with nucleic acid probes. However, libraries constructed in λgt10 are far better suited for this purpose. An efficient method is available to select against nonrecombinant λgt10 bacteriophages, and both the parental and recombinant λgt10 bacteriophages grow to higher titer than λgt11 and its derivatives. Furthermore, during amplification of libraries constructed in λgt11, some recombinants may express low levels of fusion proteins that either are toxic to the host cell or suppress growth of the infecting bacteriophage. Passage of cDNA libraries constructed in λgt11 can therefore result in loss of some classes of recombinants and selective amplification of others. For these reasons, λgt11 should be used to construct cDNA libraries that will only be screened with immunological probes.

For a more detailed description of λgt11, see Chapter 2, page 2.43.

λORF8

cDNAs inserted into λgt11 have only one chance in six of being expressed in bacteria: Only one half of the cDNA molecules will be inserted into the vector in the correct orientation with respect to the *lacZ* promoter, and only one in three of the molecules inserted in the right orientation will be in the correct reading frame to generate a fusion protein. Furthermore, because λgt11 does not require a fragment of foreign DNA to be inserted to generate a genome of packageable size, cDNA libraries constructed in this vector may contain a high proportion of nonrecombinant bacteriophages. Since no genetic selection exists to suppress the growth of such nonrecombinant bacteriophages, the number of plaques that must be screened for their ability to react with antibody specific to the protein of interest is often 10- to 20-fold higher than the number predicted from estimations of the relative abundance of the specific mRNA.

This problem can be alleviated to some extent by ensuring that the cDNA is inserted into the bacteriophage λ vector in the correct orientation for expression. Although directional cloning is not possible with λgt11, which carries identical termini on the left and right arms, a vector (λORF8) has recently been developed that has left and right arms carrying incompatible termini (Meissner et al. 1987). By equipping each end of the cDNA with a different restriction site (see below), it is possible to force the cDNA to insert into the vector in the correct orientation for expression. The inability of the arms of the vector to ligate to one another provides further advantages: The possibility of re-forming nonrecombinant genomes during the ligation reaction is minimized, and the ratio of vector to cDNA in the ligation reaction can be increased to ensure capture of cDNA molecules. The genotype of λORF8 is:

λ*sbh*Iλ1° < *lacY lacZ sr*I *lacZ*° pUC8 polycloning sites $lacI^{q1}$ *amp* > *sbh*Iλ3° *int*am *sr*Iλ3° *imm*21 *sr*Iλ4° *nin*5 *shn*dIIIλ6° *sr*Iλ5°

λORF8 carries a polycloning site (*Hin*dIII/*Bam*HI/*Eco*RI) at codons 5–10 of the *lacZ* gene (see map of λORF8 in Chapter 2, page 2.50). Insertion of cDNA into these sites frequently (but not always [Meissner et al. 1987]) results in the synthesis of inactive β-galactosidase. Consequently, most recombinant λORF8 bacteriophages produce colorless or pale blue plaques when plated on 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) indicator plates, whereas nonrecombinants always give rise to dark blue plaques.

The procedure for cloning cDNA into λORF8 is outlined in Figure 8.13. Blunt-ended cDNA, synthesized as described in the protocols section of this chapter, is treated with site-specific methylases to protect potential sites of cleavage with the restriction enzymes *Bam*HI and *Hin*dIII. Because M.*Hin*dIII methylase is not commercially available, the cDNA is methylated with M.*Bam*HI methylase and M.*Alu*I methylase (which protects against cleavage by *Hin*dIII [McClelland and Nelson 1985]). This procedure leads to two possible problems: (1) Recognition sites for *Alu*I occur frequently in DNA, and the methylation reaction must work very efficiently to ensure protection of the subset of *Alu*I sites (~1 in 16) that are cleaved by *Hin*dIII, and (2) DNA methylated by M.*Alu*I methylase may be cleaved by a restriction system

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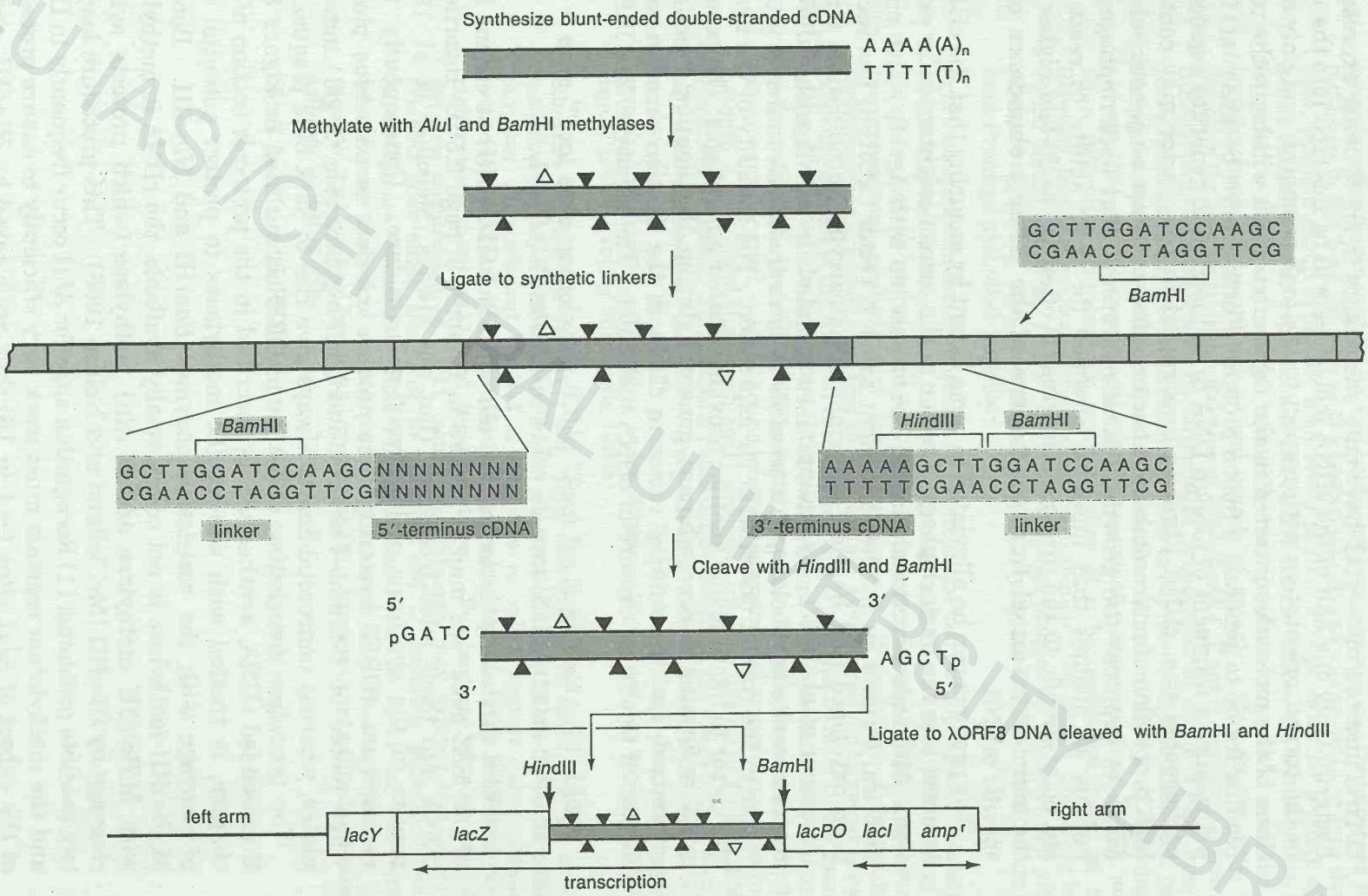

FIGURE 8.13
Directional cloning of cDNA into λORF8.

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encoded by the *mcrB* locus of *E. coli* (Raleigh and Wilson 1986). Although there is no direct evidence that this restriction system is, in fact, harmful, it is probably prudent to amplify cDNA libraries constructed in this way in a strain of *E. coli* carrying an *mcrB* mutation (e.g., MBM7014.5; see Appendix A).

When synthetic oligonucleotide linkers are ligated to the cDNA, an unmethylated *Hin*dIII site is formed by fusion of the poly(dT:dA) tract at the 3′ terminus of the cDNA to the first linker (see Figure 8.13). Addition of linkers to the 5′ terminus of the cDNA does not usually create a new restriction site but results in the formation of a tandem series of unmethylated *Bam*HI sites. Cleavage of the resulting molecules with *Bam*HI and *Hin*dIII yields cDNAs that can be inserted into compatible cloning sites located in the *lacZ* gene carried within the vector λORF8. The cDNA is therefore oriented so that transcription of its coding strand is controlled by upstream elements of the *lac* operon. The resulting mRNA would be expected to code for a fusion polypeptide that may be detected using specific antibody probes directed against the eukaryotic protein.

Although λORF8 has many attractive features, it is not free of potential problems. For example, truncated cDNAs lacking a poly(dA:dT) tract are unlikely to generate a *Hin*dIII site when synthetic linkers are added to their 3′ termini. Furthermore, cDNAs that by chance carry the sequence pTT_n at their 5′ termini will generate unmethylated *Hin*dIII sites at both their 5′ and 3′ termini. Neither class of cDNA can be cloned into λORF8 by the directional method. Finally, in comparison to λgt11, λORF8 has not been used extensively as a vector. (λgt11 has already yielded clones corresponding to an extraordinarily large and diverse range of eukaryotic genes.)

Note: For a more detailed description of λORF8, see Chapter 2, page 2.51.

λgt18, λgt19

Bacteriophage λ vectors λgt18 and λgt19 (Han et al. 1987) are derivatives of λgt11 that have been modified to carry a polycloning site at the single *Eco*RI site of λgt11 (see map of λgt18 in Chapter 2, page 2.44). They are identical to each other except for the orientation of the polycloning site and can therefore be used for nondirectional and directional cloning of cDNA. In addition, at least one of the sites in the polycloning region (*Sal*I) occurs quite rarely in mammalian DNA (once every 100 kb on average), and so there is little possibility that a moderately sized clone of cDNA will contain a *Sal*I site. Consequently, blunt-ended double-stranded cDNAs carrying *Sal*I linkers need not be protected by methylation before they are digested with the restriction enzyme. After fragments of linkers are removed, the resulting population of cDNA molecules can be ligated directly to the arms of λgt18 or λgt19. Note that, as in the case of λgt11, cDNAs inserted into λgt18 and λgt19 in this way have only one chance in six of being expressed in bacteria: Only one half of the cDNA molecules will be inserted into the vector in the correct orientation with respect to the *lacZ* promoter, and only one in three of the molecules inserted in the right orientation will be in the correct reading frame to generate a fusion protein. The genotype of λgt18 and λgt19 is:

*λlac*5 Δ*shn*dIIIλ2-3 *sr*Iλ3° Δ*ssl*Iλ1-2 *cIts*857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

Note: For a more detailed description of λgt18 and λgt19, see Chapter 2, page 2.45.

λgt20, λgt21

λgt20 and λgt21 were derived from λgt18 and λgt19, respectively (Han and Rutter 1988), and thus have many of the same properties described above for λgt18 and λgt19. Two specific alterations were made in λgt20 and λgt21: (1) A synthetic *chi* site was introduced to eliminate the selection of cDNA inserts containing *chi* sites (see Chapter 2), and (2) the *Sac*I and *Xba*I sites were eliminated from the vector arms so that the *Xba*I site in the polycloning site is unique (see map of λgt20 in Chapter 2, page 2.46). These manipulations resulted in deletion of about 500 bp in the bacteriophage λ DNA to the right of *lac*5. This allows larger inserts (up to 8.2 kb) than in the λgt18 and λgt19 vectors. The genotype of λgt20 and λgt21 is:

*λlac*5 Δ*sx*Iλ1° *chi* Δ*shn*dIIIλ2-3 *sr*Iλ3° Δ*ssl*Iλ1-2 *cIts*857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

Note: For a more detailed description of λgt20 and λgt21, see Chapter 2, page 2.47.

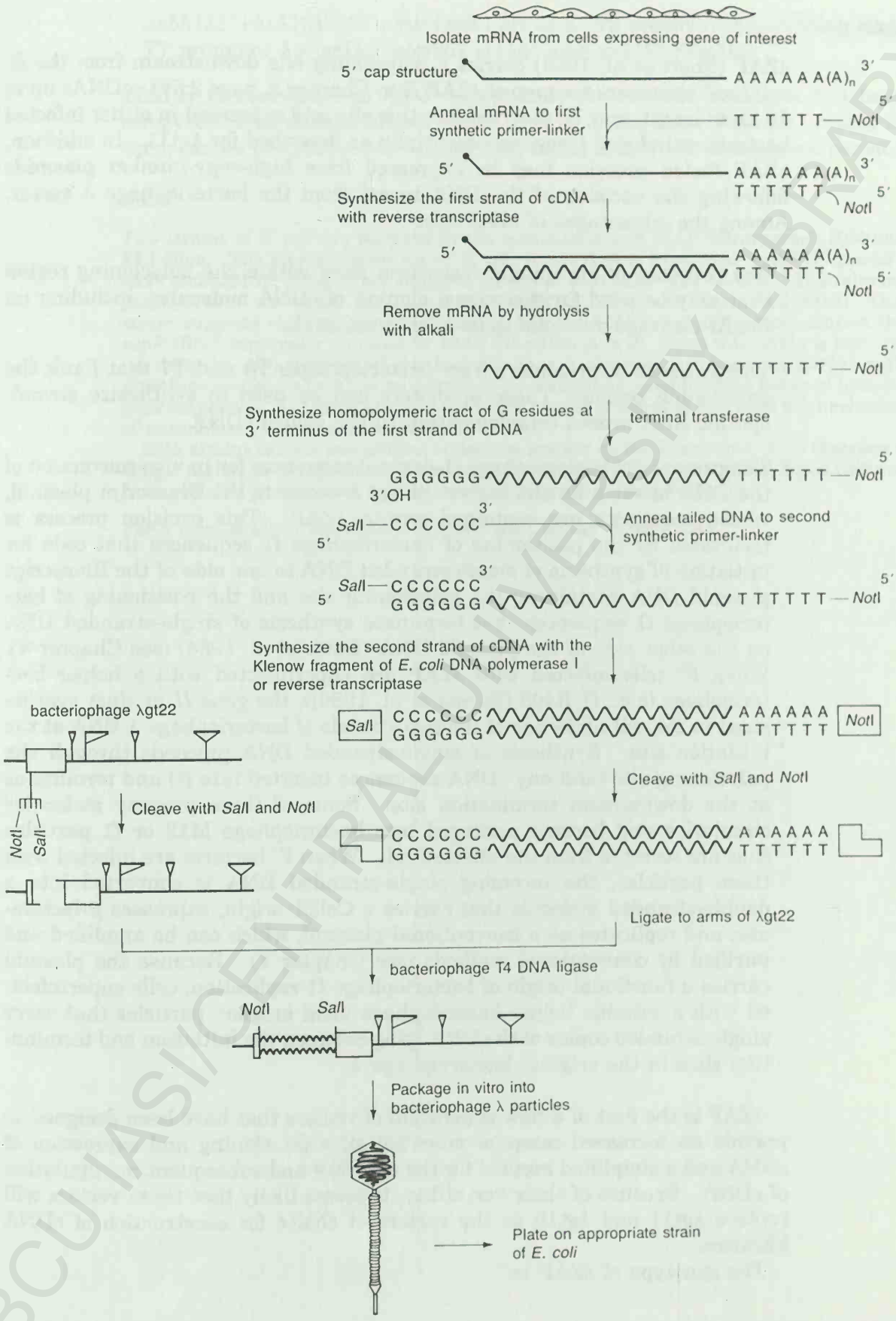
λgt22, λgt23

Bacteriophage λ vectors λgt22 and λgt23 (Han and Rutter 1987) are derivatives of λgt18 and λgt19 that carry a synthetic *chi* sequence (Smith et al. 1981) and a different in-frame polycloning site near the 3′ terminus of the coding region of *lacZ* (see map of λgt22 in Chapter 2, page 2.48). λgt22 and λgt23 are identical to each other except for the orientation of the polycloning site. The *Xba*I and *Sac*I sites of λgt18 and λgt19 arms have been removed, leaving vectors that contain five unique restriction sites for cloning: *Not*I, *Xba*I, *Sac*I, *Sal*I, and *Eco*RI. (In early versions of these vectors, there was an additional *Sac*I site in *lacZ*.) Up to 8.2 kb of cloned cDNA inserted at any of these sites can be expressed as a LacZ fusion protein. λgt22 and λgt23 are designed to allow directional cloning of cDNA into the *Sal*I and *Not*I sites of the polycloning site. The syntheses of the first and second strands of cDNA are primed by primer-linkers (Figure 8.14), and the resulting double-stranded cDNA is digested with *Sal*I and *Not*I and ligated directly into the arms of the vector. Because these restriction enzymes cleave mammalian DNA so rarely, there is no need to protect the cDNA from digestion. The sequential use of different primer-linkers for the synthesis of the first and second strands of cDNA allows the double-stranded cDNA to be inserted in only one orientation with respect to the *lacZ* promoter. One in three of the resulting recombinants would be expected to carry a cDNA molecule in the correct reading frame to generate a fusion protein. The genotype of λgt22 and λgt23 is:

*λlac*5 Δ*sx*Iλ1° *chi* Δ*shn*dIIIλ2-3 *sr*Iλ3° Δ*ssl*Iλ1-2 *cIts*857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

Note: For a more detailed description of λgt22 and λgt23, see Chapter 2, page 2.49.

FIGURE 8.14 (See facing page.)
Cloning cDNA with primer-linkers.


Isolate mRNA from cells expressing gene of interest
5′ cap structure
A A A A A A(A)n 3′
Anneal mRNA to first synthetic primer-linker
T T T T T T— NotI 5′
Synthesize the first strand of cDNA with reverse transcriptase
Remove mRNA by hydrolysis with alkali
Synthesize homopolymeric tract of G residues at 3′ terminus of the first strand of cDNA
terminal transferase
G G G G G G
3′OH
SalI—C C C C C C
Anneal tailed DNA to second synthetic primer-linker
Synthesize the second strand of cDNA with the Klenow fragment of E. coli DNA polymerase I or reverse transcriptase
SalI
NotI
bacteriophage λgt22
Cleave with SalI and NotI
Ligate to arms of λgt22
bacteriophage T4 DNA ligase
Package in vitro into bacteriophage λ particles
Plate on appropriate strain of E. coli

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λZAP (Short et al. 1988) carries a polycloning site downstream from the *E. coli lacZ* promoter (see map of λZAP/R in Chapter 2, page 2.52). cDNAs up to 10 kb in length may be inserted into this site and expressed in either infected bacteria or induced lysogens, essentially as described for λgt11. In addition, λZAP fusion proteins may be expressed from high-copy-number plasmids following the excision of the DNA insert from the bacteriophage λ vector. Among the advantages of λZAP are:

- The presence of six unique restriction sites within the polycloning region that may be used for directional cloning of cDNA molecules, including an *Eco*RI cleavage site analogous to that of λgt11.
- The presence of promoters from bacteriophages T3 and T7 that flank the polycloning region. These promoters can be used to synthesize strand-specific RNA probes complementary to the cloned cDNA.
- The presence of bacteriophage-f1-derived sequences for in vivo conversion of the DNA insert from the bacteriophage λ vector to the Bluescript plasmid, whose sequences are contained within λZAP. This excision process is facilitated by the positioning of bacteriophage f1 sequences that code for initiation of synthesis of single-stranded DNA to one side of the Bluescript plasmid DNA containing the polycloning site and the positioning of bacteriophage f1 sequences that terminate synthesis of single-stranded DNA on the other side of the plasmid DNA (Dotto et al. 1984) (see Chapter 4). When F′ cells infected with λZAP are superinfected with a helper bacteriophage (e.g., f1 R408 [Russel et al. 1986]), the gene *II* product synthesized in the cell nicks one of the two strands of bacteriophage λ DNA at the initiation site. Synthesis of single-stranded DNA proceeds through the polycloning site (and any cDNA sequences inserted into it) and terminates at the downstream termination site. Some of these progeny molecules circularize and become packaged into bacteriophage M13 or f1 particles that are secreted from the infected cell. When F′ bacteria are infected with these particles, the incoming single-stranded DNA is converted into a double-stranded molecule that carries a ColE1 origin, expresses β-lactamase, and replicates as a conventional plasmid, which can be amplified and purified by conventional methods (see Chapter 1). Because the plasmid carries a functional origin of bacteriophage f1 replication, cells superinfected with a suitable helper bacteriophage yield progeny particles that carry single-stranded copies of the DNA lying between the initiation and termination sites in the original bacteriophage λ.

λZAP is the first of a new generation of vectors that have been designed to provide an increased range of potential sites for cloning and expression of cDNA and a simplified method for the recovery and subsequent manipulation of cDNA. Because of their versatility, it seems likely that these vectors will replace λgt11 and λgt10 as the vectors of choice for construction of cDNA libraries.

The genotype of λZAP is:

λ*sbh*Iλ1° *chi*A131 <T *amp* ColE1 *ori lacZ′* T3 promoter–polycloning site–T7 promoter I> *sr*Iλ3° *cIts*857 *sr*Iλ4° *nin*5 *sr*Iλ5° *S*am100

λZAPII is equivalent to λZAP except that the *S*am100 mutation has been removed. This change allows better growth of the bacteriophage, which, in turn, causes the plaques to become blue much sooner (H. Short, pers. comm.).

Notes

Two strains of *E. coli* are supplied by the manufacturer of λZAP (Stratagene): BB4 and XL1-Blue. The relevant genotype of *E. coli* strain BB4 is *supE supF hsdR* [F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]. This strain supports growth of both λZAP and λZAPII. The relevant genotype of *E. coli* strain XL1-Blue is *supE hsdR lac*$^-$ [F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]. This strain supports vigorous growth of λZAPII, but not λZAP, since it does not contain the *supF* tRNA suppressor required for λZAP (*S*am100) growth. Since this strain is Rec$^-$, its use may be advantageous if the bacteriophage λ clone is carrying rare cDNAs with repetitive sequences that can be deleted or rearranged by recombination between homologous sequences. XL1-Blue also has the added benefit of allowing improved color selection of recombinant versus nonrecombinant clones.

Both strains carry a tetracycline resistance marker on the F′ episome. It is therefore a simple matter to ensure that the bacteria are expressing F pili by starting liquid cultures from colonies grown on an agar plate containing 15 μg/ml tetracycline.

For a more detailed description of λZAP, see Chapter 2, page 2.53.

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IDENTIFICATION OF cDNA CLONES OF INTEREST

Methods of Screening

There are three methods to screen cDNA libraries for clones of interest:

- Nucleic acid hybridization
- Immunological detection of specific antigens
- Sib selection either by hybrid selection and translation of mRNA or by production of biologically active molecules

Most cloning projects today are aimed at isolating cDNAs corresponding to rare mRNAs and therefore require screening of large numbers of recombinant clones. This can be carried out effectively with only two types of reagents: antibodies and nucleic acid probes. In those rare instances when both types of reagents are available, nucleic acid probes are preferred because they can be used under a variety of different stringencies that minimize the chance of undesirable cross-reactions. Furthermore, nucleic acid probes will detect all clones that contain cDNA sequences, whereas antibodies will react only with a subset of these clones (in some cases one in six at best) in which the cDNA has been inserted into the vector in the correct reading frame and orientation. cDNA libraries that are to be screened by antibodies therefore need to be larger (by a factor of at least 6) than those that are to be screened by nucleic acid probes. Consequently, when using antibody probes to search for a cDNA clone corresponding to a mammalian mRNA present at the level of 1 molecule/cell or less, it is desirable to construct cDNA expression libraries that contain in excess of 10^7 members. This is not easy, especially when the amounts of mRNA are limited. Furthermore, screening a library of this size is expensive and laborious, and it becomes worthwhile to explore methods to enrich the mRNA (or cDNA derived from it) for the sequences of interest (see pages 8.6–8.10).

NUCLEIC ACID HYBRIDIZATION

This is the most commonly used and reliable method of screening cDNA libraries for clones of interest. None of the other methods displays such an abundance of attractive features. Screening by nucleic acid hybridization allows extremely large numbers of clones to be analyzed simultaneously and rapidly, does not require that the cDNA clones be full-length, and does not require that an antigenically or biologically active product be synthesized in the host cell. Furthermore, as a result of more than 20 years of work, the theoretical basis of nucleic acid hybridization is well-understood. This has led to the development of a large number of different techniques that can accommodate nucleic acid probes of very different lengths and specificities. Details of the methods for the preparation and use of these probes are presented in Chapters 10 and 11.

Homologous probes

Homologous probes contain at least part of the exact nucleic acid sequence of the desired cDNA clone. They are used in a variety of circumstances, for

example, when a partial clone of an existing cDNA is used to isolate a full-length clone from a cDNA library. Usually, a fragment derived from one end or the other of the existing clone is isolated, radiolabeled in vitro, and used to probe a library. Hybridization with homologous probes is always carried out under stringent conditions.

Partially homologous probes

Partially homologous probes are used to detect cDNA clones that are related, but not identical, to the probe sequences. If neither antibody nor nucleic acid probes are available, a number of alternative strategies can be considered. For example, if the same gene has already been cloned from another species or if a related gene has been cloned from the same species, it would be worthwhile carrying out a series of trial experiments to determine whether there is sufficient conservation of nucleic acid sequence to allow the screening of a cDNA library by hybridization. This is most easily accomplished by performing a series of Southern and northern hybridizations at different stringencies. For example, a large batch (50 μg) of genomic DNA is cleaved with a restriction enzyme that cleaves the probe sequence at one or two well-separated sites. It is a good idea to digest an equal amount of genomic DNA of the original species for use as a positive control. Aliquots (5–10 μg) of the digests are then applied to adjacent slots of a 0.8% agarose gel, electrophoresis is carried out, and the fragments are then transferred to a nitrocellulose filter as described in Chapter 9, pages 9.34–9.41. The filter is cut into strips, each of which is hybridized under different conditions to identical amounts of radioactive probe. For aqueous hybridization, the ionic strength of the solution is kept constant (usually 1 M Na^+) while the temperature of annealing is progressively lowered (from 68°C to 42°C). The strips are then washed extensively at the temperature of hybridization with a solution containing 2 × SSC, 0.5% SDS. When hybridization is carried out in solvents containing formamide, the temperature and ionic strength are usually kept constant (42°C and 6 × SSC [or 6 × SSPE], respectively) while the amount of formamide in the annealing buffer is progressively lowered from 50% to 0%. The strips are then washed extensively at 50°C in 6 × SSC, 0.5% SDS. A similar series of hybridizations can be carried out with mRNA preparations that have been fractionated by electrophoresis and transferred to a solid support. In both cases, the aim is to establish conditions that will allow the previously cloned gene to be used as a probe for the cDNA of interest, without undue interference from background hybridization.

Total cDNA probes

Total cDNA probes are prepared by uniform incorporation of radiolabeled nucleotides with reverse transcriptase or end-labeling of total or fractionated poly(A)$^+$ mRNA. They can be used to screen libraries of cDNA for specific clones *if* the cDNA clones of interest correspond to mRNA species present in the initial population at a frequency of at least 1 in 200 (see Gergen et al. 1979; Dworkin and Dawid 1980). It is not possible to detect cDNA clones homologous to species that are represented rarely in the mRNA preparation.

Subtracted cDNA probes

Subtracted cDNA probes are often used to probe cDNA libraries for clones that correspond to mRNAs that are differentially regulated. A cDNA probe prepared from one type of mRNA is depleted of sequences that are present in a second type of mRNA by subtractive hybridization (Timberlake 1980; Zimmerman et al. 1980). Typically, the cDNA is hybridized two or three times in succession to a 20-fold excess of the second mRNA, and the cDNA:mRNA hybrids are removed by chromatography on hydroxyapatite. The unhybridized cDNA is then annealed to a 100-fold excess of the mRNA preparation from which it was originally synthesized, and the resulting cDNA:mRNA hybrids are this time recovered by chromatography on hydroxyapatite. After the mRNA is removed by alkaline hydrolysis, the cDNA, which is highly enriched for sequences specific to the original mRNA, is used to probe a cDNA library for clones homologous to these sequences.

Subtracted cDNA probes are particularly valuable when there are very few differences between the two starting mRNA preparations, i.e., when most species of mRNA are represented equally in the two preparations and a small proportion (<2%) of the mRNAs are not present at all in one preparation. cDNAs that have been cloned using subtracted cDNA probes include the murine J immunoglobulin chain (Mather et al. 1981) and the murine T-cell receptor (Hedrick et al. 1984).

A slightly different approach is used when two preparations of mRNA share sequences that are present at different concentrations. Examples of such sib pairs might be mRNAs extracted from control cells and cells that have been exposed to heat shock, drugs, or hormones. cDNAs corresponding to mRNAs whose expression is altered by such treatments can often be detected by *differential hybridization*. ^{32}P-labeled first-strand cDNAs are synthesized in vitro using both mRNAs as templates. Most of the cDNA sequences correspond to mRNAs whose concentrations are not appreciably changed by the treatment to which the cells were exposed. However, a minority of the cDNAs will be copied from mRNAs whose concentrations are significantly increased or decreased. The two cDNA probes are then used to screen replicas of a cDNA library constructed from mRNA extracted from control cells (when searching for mRNAs that are repressed) or treated cells (when searching for mRNAs that are induced). The clones that hybridize preferentially to one of the cDNA probes are chosen for further analysis. Among the many examples of genes cloned in this way are the galactose-inducible genes of yeast (St. John and Davis 1979), human fibroblast interferon (Taniguchi et al. 1980), the glucose-regulated proteins of mammalian cells (Lee et al. 1981), growth-related proteins (Foster et al. 1982; Cochran et al. 1983; Linzer and Nathans 1983), differentiation-specific proteins (Spiegelman et al. 1983), and a variety of heat-shock proteins and stress proteins (see, e.g., Mason et al. 1986). The procedure has also been used to identify cDNA clones of developmentally regulated mRNAs from organisms of many different species including *Xenopus* (Williams and Lloyd 1979; Weeks et al. 1985), *Dictyostelium* (Rowekamp and Firtel 1980; Mehdy et al. 1983), sea urchins (Lasky et al. 1980), and mice (Gorman et al. 1985).

Synthetic oligonucleotide probes

Synthetic oligonucleotide probes are tracts of dNTPs of defined sequence that have been synthesized in vitro. The sequence of these probes is deduced, using the genetic code, from short regions of the known amino acid sequence of the protein of interest. Because of the degeneracy of the genetic code, it is very unlikely that a given sequence of amino acids will be specified by a predictable single oligonucleotide of defined sequence. Instead, in the vast majority of cases, the same sequence of amino acids can be specified by many different oligonucleotides. There is no way to know with certainty which of these oligonucleotides is actually used in the gene of interest. Three solutions have been found to this problem:

1. A family of oligonucleotides can be synthesized containing all possible sequences that can code for a given sequence of amino acids. The number of members in this family depends on the degree of degeneracy of the codons for the particular amino acids. However, since all possible oligonucleotide sequences are represented, at least one of the members will match perfectly with the cDNA clone of interest. To keep the size of each family within manageable proportions, short oligonucleotides (14–17 nucleotides) are generally used—the minimum size that is practical for hybridization. Often, more than one family of oligonucleotides is synthesized based on separate sequences of amino acids.

2. A longer (40–60-base) oligonucleotide of unique sequence can be synthesized using the most commonly used codon for each amino acid. (Avoid using the dinucleotide CpG, since it is underrepresented in most eukaryotic DNAs.) Almost certainly, this oligonucleotide will not match exactly the sequence in the cDNA, but it will fit well enough to be detected by hybridization under nonstringent conditions.

3. An oligonucleotide can be synthesized that contains a base such as inosine at positions of high potential degeneracy. Inosine can pair with all four conventional bases without seriously compromising the stability of the resulting hybrid. It is therefore possible to generate families of longer oligonucleotides that are reduced in number and yet are capable of hybridizing to virtually all cDNA clones that are likely to code for the protein of interest.

Finally, if the protein sequence available is from the amino terminus of the protein, the cDNA library that is to be screened must be of high quality to ensure that most of the 5′ terminus of the mRNA is represented. For a detailed discussion of synthetic oligonucleotide probes, see Chapter 11.

IMMUNOLOGICAL DETECTION OF SPECIFIC ANTIGENS

cDNA libraries constructed in expression vectors such as λgt11, λgt18–23, λZAP, and λORF8 can be screened with antibody directed against the protein of interest (see Chapter 12 for experimental details). Nitrocellulose filters imprinted with the detritus of bacterial lysis are soaked in a solution

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containing the antibody. After washing, the filter is incubated with *Staphylococcus aureus* protein A or with a second antibody directed against the species-specific epitopes of the first antibody. In the original descriptions of the method, the secondary ligand was radiolabeled with ^{125}I. Today, the secondary ligand is covalently linked to an enzyme whose activity can be detected histochemically (e.g., alkaline phosphatase).

The key to success with this method lies in the quality of the antibody. It is essential that the antibody efficiently recognize the denatured protein (i.e., it should produce strong signals on western blots). Screening is made easier and more sensitive if, in addition, the antibody is derived from a polyclonal antiserum of high titer. Because such antisera normally react with many different epitopes, the chances of detecting a cDNA clone that expresses a fragment of the protein of interest are increased. Polyclonal antisera often contain cross-reacting antibodies that recognize nonrecombinant components of the bacterial lysate; these must be removed before screening is undertaken (see Chapter 12).

The background of nonspecific binding is much lower when a monoclonal antibody is used as a probe. However, the number of recombinants that can be detected is also reduced, because each individual monoclonal antibody can react with only a single epitope. The ideal immunological probe might therefore consist of a cocktail of several different monoclonal antibodies, each of which reacts strongly with denatured protein.

SIB SELECTION OF cDNA CLONES

Two methods of screening are based on the concept of dividing a large cDNA library into a manageable number of pools, each consisting of between 10 and 100 clones. These pools are then tested for the sequence of interest. The lower complexity of each pool allows methods that are relatively insensitive to be applied to a complete cDNA library. After a pool is identified that scores positively, it is subdivided into successively smaller and smaller pools, each of which is retested until the cDNA clone of interest is isolated. The process of sib selection and analysis can sometimes be performed more rapidly if, during subdivision of the initial pool, the individual clones are assigned to their subpools in a matrix format (Wong et al. 1985). This expands the number of subpools by a factor of 2 or 3, but it often leads directly to the identification of the clone of interest.

Hybrid selection

In this method, cDNA clones carrying sequences complementary to specific mRNAs are denatured, immobilized on a solid matrix, and hybridized to preparations of mRNA. The mRNA:cDNA hybrids are then heated to release the mRNA, which is then translated in a cell-free protein-synthesizing system or in *Xenopus* oocytes. The translation products are identified by immunoprecipitation and/or SDS-polyacrylamide gel electrophoresis (see Chapter 18).

Today, this procedure is used mainly to confirm the identification of cDNA clones isolated by other means. However, before the development of methods to screen cDNA libraries with immunological or oligonucleotide probes, it was

also used occasionally for the primary isolation of cDNA clones. For example, Parnes et al. (1981), using DNA from pools that contained as many as 100 individual cDNAs cloned in plasmids, were able to detect specific translation of an mRNA that represented approximately 0.03% of the total cellular mRNA. This entire procedure is labor-intensive and is worth attempting only as a last resort with mRNAs that can be greatly enriched by size selection and for which no other probes are available.

Production of biologically active molecules

A few groups have used sib selection and analysis to screen cDNA libraries for the production of biologically active protein molecules in cells. For example, in vitro assays have been used to identify cDNA clones that express the human lymphokines colony-stimulating factor and interleukin-3 in cultures of mammalian cells (Wong et al. 1985; Yang et al. 1986). This approach is usually undertaken when no other methods of screening are available and when the protein product is small enough to give reasonable assurance that the cDNA library will contain full-length clones.

Methods to Validate Clones of cDNA

cDNA libraries are usually plated at high density for screening with antibody or nucleic acid probes, and any clones that react positively in the first round require several additional cycles of plating and screening before they can be considered pure. However, the ability to react consistently with a particular probe, although an encouraging and necessary property, is not sufficient to prove that a given cDNA clone is derived from the mRNA of interest. The only absolute proof of identity is to show that the cDNA clone contains an open reading frame that codes for the entire amino acid sequence of the protein. Since this is clearly impractical for most proteins of current interest, other, less elemental tests must be used. In decreasing order of rigor, these include:

- Expression from the full-length cDNA in prokaryotic or eukaryotic cells of a protein that displays the correct biological or enzymatic activity.
- Correspondence between portions of the nucleotide sequence of the cDNA and the amino acid sequences of peptides derived from the purified protein.
- Correspondence between the peptide maps of the polypeptide synthesized in vitro by transcripts of the cDNA clone and peptide maps of the authentic protein.
- Immunoprecipitation of the polypeptide synthesized in vitro or in vivo from transcripts of the cDNA clone by antibodies raised against the protein of interest. The stringency of this test increases when it is carried out with a series of monoclonal antibodies that recognize different epitopes on the protein.
- Immunoprecipitation of the authentic protein with antibodies raised against synthetic peptides whose sequences are determined by the nucleic acid sequence of the cloned cDNA.

It is essential that the tests used to validate a cDNA clone do not use the same reagents that were used to identify the clone in the first place. If, for example, a clone is selected by screening a library with antibody, it cannot be validated in tests that use the same antibody (such as immunoprecipitation of polypeptides synthesized in vitro from transcripts of the clone). In addition, the cloned cDNA should hybridize to mRNA whose size, tissue, and species distribution are not inconsistent with the structure and function of the protein. Finally, the properties of the polypeptide predicted by the nucleotide sequence of the cDNA should be in accord with those of the authentic protein. For example, the vast majority of secreted or plasma membrane proteins are derived from polypeptide precursors that carry a hydrophobic signal sequence at their amino termini. The absence of such a signal from the predicted polypeptide would therefore be cause for concern. Similarly, the predicted size of the polypeptide, its overall charge and amino acid composition, the number of potential glycosylation sites, and the presence of hydrophobic regions that could serve as transmembrane anchors should be consistent with the known properties of the authentic protein.

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In the remainder of this chapter, we describe in detail the most effective methods to construct cDNA libraries in bacteriophage λ vectors. The scheme is based on the procedures of Gubler and Hoffman (1983) and Huynh et al. (1985) and has become the standard procedure used to synthesize and clone double-stranded cDNA. The basic steps in this scheme are:

1. Oligo(dT)-primed synthesis of the first strand of cDNA with reverse transcriptase
2. Synthesis of the second strand of cDNA (using RNAase H and *E. coli* DNA polymerase I), including the repair reaction with bacteriophage T4 polynucleotide kinase and *E. coli* DNA ligase
3. Methylation of *Eco*RI sites
4. Ligation to phosphorylated *Eco*RI linkers and digestion of linkers to yield cohesive termini
5. Size selection of cDNA
6. Ligation to bacteriophage λ arms, packaging of DNA into bacteriophage λ particles, and test plating on *E. coli*
7. Analysis of cDNA inserts (bacteriophage λ minipreps)
8. Generation of a complete cDNA library
9. Amplification of cDNA libraries

Virtually all of these steps can be monitored by control reactions. Furthermore, because these methods have been in common use for several years, most of the problems have been uncovered and solved. However, with the recent development of bacteriophage λ vectors with *Not*I or *Sal*I sites, it is often possible to simplify the basic scheme outlined above by using *Not*I or *Sal*I linkers instead of *Eco*RI linkers. Because the chance is small that the cDNA of interest will be cleaved by such enzymes, the methylation reaction with M.*Eco*RI methylase may be eliminated. The modifications that permit the use of *Not*I or *Sal*I linkers are marked at the appropriate points in the description of the basic protocol.

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Precautions

1. Making cDNA libraries is an expensive and time-consuming endeavor. To minimize the possibility of mistakes, many laboratories prepare ahead of time a set of reagents, buffers, and substrates that are used for no other purpose than cDNA cloning. This eliminates delays between one step in the protocol and the next and reduces the chance of accidental contamination by nucleases or exogenous nucleic acids. There are many anecdotes of cDNA libraries containing DNA sequences that had their origin in restriction enzymes and solutions that had been contaminated by fellow laboratory workers. cDNA cloning is particularly vulnerable to such contamination because it involves the manipulation of small amounts of cDNA through a succession of enzymatic reactions. The way that these reactions are arranged can lead to the efficient salvage of contaminating DNA. For convenience, a list of the critical materials, reagents, and buffers that are used to prepare and clone cDNA are presented in Table 8.1. Whenever possible, the solutions should be sterilized by autoclaving.

2. Carry out all operations in sterile microfuge tubes, using sterile pipettes and pipette tips.

3. In such a long series of enzymatic reactions and manipulations, there are many points at which potential problems may arise. However, by holding firmly to the rule of checking the products of each reaction, it is almost always possible to avoid ruination. Three commonly used routines for minimizing difficulties are:

   - *Checking the products of each reaction before proceeding to the next.* Although attractive in theory, this method of procedure slows down cDNA cloning to an unacceptable rate. However, there are several critical steps in the protocol at which we strongly recommend that checks (which are described at the appropriate points in the text) be carried out before proceeding further. These are:

Step	Check
Synthesis of the second strand of cDNA	Size and yield of product
Methylation of double-stranded DNA	Resistance of DNA to subsequent cleavage
Digestion of linkers with restriction enzymes	Cleavage of unmethylated test DNA
Ligation of cDNA to bacteriophage λ arms	Test ligation/packaging

   Small samples of every reaction should be saved for later analysis, if it should become necessary to pinpoint where problems may have occurred.

   - *Setting up a series of small-scale pilot reactions 1 or 2 days ahead of the time that these reactions will be used to synthesize the main batch of cDNA.* This procedure allows any potential problems to be identified and solved without putting the entire experiment at risk.

   - *Carrying out two sets of full-scale reactions, the second of which is begun 2 days after the first.* The second batch of cDNA acts as insurance

against unexpected loss of the first and guarantees that no more than 2 days will be lost.

4. Most losses of cDNA occur during precipitation with ethanol. Taking the following precautions helps to avoid this problem:

   - After adjusting the salt concentration of the DNA solution to 0.3 M sodium acetate or 2 M ammonium acetate and adding 2 volumes of ethanol, mix the contents of the tube very well. Stand the tightly closed tube at room temperature for at least 15 minutes, and then centrifuge at 12,000*g* for at least 15 minutes at 4°C. Under these conditions, even very small quantities of DNA (10 ng) are precipitated and there is no need to use a carrier.

     Some workers prefer to freeze the ethanolic mixture at −70°C (in a dry-ice/ethanol bath) for 15 minutes or to store it for more prolonged periods at −20°C before centrifugation. In our hands, these procedures do not improve the efficiency with which DNA is precipitated by ethanol.

   - Save the ethanol solution until you are sure that all of the DNA has been recovered. This is especially important after precipitates of DNA have been washed with 70% ethanol—treatment that often loosens the precipitates from the wall of the tube.

   - Make sure you redissolve all of the cDNA after ethanol precipitation. The precipitated DNA is not all found at the bottom of the tube—about 30–40% is plastered on the wall. This DNA is redissolved by rolling a bead of solvent several times over the appropriate segment of the wall. Use a pipette tip to roll the bead of solvent. Check that no detectable radioactivity remains in the tube after the redissolved cDNA has been removed.

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TABLE 8.1 Reagents Used to Synthesize and Clone cDNA

Actinomycin D	Required only if self-priming of second-strand cDNA synthesis in unacceptably high (see note to step 5g on page 8.65). Preparations of actinomycin D supplied by pharmaceutical manufacturers for therapeutic uses often contain additional substances such as sugars and salts. Such preparations can be used to suppress self-priming as long as the concentration of actinomycin D is verified by measuring the absorbance of the stock solution at 440 nm. The molar extinction coefficient of pure actinomycin D (m.w. = 1255) in aqueous solution is 21,900. The absorbance at 440 nm of a solution containing 1 mg/ml of the drug is therefore 0.182. Stock solutions of actinomycin D are stored at −20°C in foil-wrapped tubes. **Caution:** Actinomycin D is a teratogen and carcinogen. Stock solutions should be prepared, wearing gloves and a mask, in a chemical hood, not on an open bench.
S-Adenosyl-L-methionine	Iodide salt, grade I. Make a stock solution (20 mM) in 5 mN H_2SO_4, 10% ethanol. Store in aliquots at −20°C. New England Biolabs provides a solution (30 mM) of *S*-adenosyl-L-methionine when you buy M.*Eco*RI methylase.
[α-^{32}P]dCTP	400 Ci/mmole, 10 μCi/μl in aqueous solution. [α-^{32}P]dATP and [α-^{32}P]dTTP should not be used as radioactive tracers to monitor synthesis of the first and second strands of cDNA, since they may be incorporated preferentially into the cDNA derived from the poly(A)$^+$ tract at the 3′ terminus of the mRNA.
ATP	Make 50 mM and 10 mM stock solutions as described for dNTPs. Store in aliquots at −70°C.
Bacteriophage λ arms	If you plan to make a large number of libraries, it is much cheaper to prepare arms by agarose gel electrophoresis (see Chapter 2) than to purchase them. For occasional users, dephosphorylated arms for some vectors are available from Stratagene. Whatever the source of arms, it is important to carry out a series of pilot reactions to check that they can be ligated to foreign DNA and packaged efficiently into infectious bacteriophage particles.
Bacteriophage T4 DNA ligase	Most manufacturers purify the enzyme from strains of *E. coli* that express the cloned gene at a high level. At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units (as defined by New England Biolabs). 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units. Bacteriophage T4 DNA ligase is supplied in concentrated solution (1–5 units/μl). This should be diluted and stored at a concentration of 100 units/ml in 20 mM Tris · Cl (pH 7.6), 60 mM KCl, 5 mM dithiothreitol, 500 μg/ml bovine serum albumin, 50% glycerol. Bacteriophage T4 DNA ligase is stable at this concentration in this buffer for 3 months at −20°C.
Bacteriophage T4 DNA polymerase	Most manufacturers purify the enzyme from *E. coli* infected with a mutant of bacteriophage T4 defective in gene *32* (Panet et al. 1973).
Bacteriophage T4 polynucleotide kinase	New England Biolabs sells enzyme isolated from a strain of *E. coli* lysogenic for a transducing bacteriophage λ that produces large amounts of bacteriophage T4 polynucleotide kinase.
β-Mercaptoethanol (0.3 M)	Make a fresh solution just before use by adding 300 μl of the 14.4 M solution supplied by the manufacturer to 14.1 ml of H_2O.

Chloroform

Deoxyribonucleoside triphosphates

Dissolve each dNTP in water at an approximate concentration of 100 mM. Using 0.05 M Tris base and a micropipette, adjust the pH of each of the solutions to 7.0 (use pH paper to check the pH). Dilute an aliquot of the neutralized dNTP appropriately, and read the optical density at the wavelengths given in the table below. Calculate the actual concentration of each dNTP. Dilute the solutions with water to a final concentration of 50 mM dNTP. Store each separately at −70°C in small aliquots.

Base	Wavelength (nm)	Extinction coefficient (ϵ) (M^{-1} cm^{-1})
A	259	1.54×10^4
G	253	1.37×10^4
C	271	9.10×10^3
T	260	7.40×10^3

For a cuvette with a path length of 1 cm, absorbance = ϵM.

100 mM stock solutions of each dNTP are commercially available (Pharmacia) if you do not want to prepare your own. Use the four stock solutions to make a single working solution containing each of the four dNTPs at a concentration of 5 mM. Store the mixture in aliquots at −70°C.

Dithiothreitol (0.1 M)

Make a stock solution in water. Store in small aliquots at −20°C.

E. coli

Use strains C600 (BNN93) for growth and BNN102 (C600*hflA*) for screening of cDNA libraries constructed in bacteriophage λgt10. Use strain Y1090*hsdR* for growth and screening of cDNA libraries constructed in bacteriophage λgt11. Use strain BB4 for growth and screening of cDNA libraries constructed in λZAP and λZAPII or strain XL1-Blue for λZAPII. (Strain XL1-Blue supports vigorous growth of λZAPII but not λZAP.)

E. coli DNA ligase

This enzyme is purified from *E. coli* by several manufacturers.

E. coli DNA polymerase I

Several manufacturers sell enzyme isolated from a strain of *E. coli* lysogenic for a bacteriophage λ that carries the gene (*polA*) that codes for the holoenzyme of *E. coli* DNA polymerase I (Murray and Kelley 1979). Most commercial suppliers sell this enzyme in buffer containing 50% glycerol. Usually, 1 μl of the preparation contains 5 units of enzyme (for a definition of a unit, see Richardson et al. 1964).

*Eco*RI

New England Biolabs sells enzyme isolated from a strain of *E. coli* that expresses the *RI* restriction and modification genes from a multicopy plasmid.

M.*Eco*RI methylase

New England Biolabs sells enzyme isolated from a strain of *E. coli* that expresses the methylase gene from a multicopy plasmid.

EDTA (0.5 M; pH 8.0)

IPTG

Isopropylthio-β-D-galactoside (m.w. = 238.3). Make a solution of IPTG by dissolving 2 g of IPTG in 8 ml of H_2O. Adjust the volume of the solution to 10 ml with H_2O and sterilize by filtration through a 0.22-micron disposable filter. Dispense the solution into 1-ml aliquots and store them at −20°C.

KCl (1 M)

Linearized plasmid DNA

This is used to check the efficiency of methylation of *Eco*RI sites (see page 8.66–8.67) and to check the efficiency of digestion following linker addition (see page 8.69). The plasmid should therefore contain at least one *Eco*RI site located some distance from the ends of the linear DNA (e.g., Xf3 or pBR322 linearized by digestion with *Pst*I). Digest 1 μg of the plasmid DNA to completion with *Pst*I. Purify the DNA by extraction with phenol:chloroform and precipitation with ethanol. Redissolve the DNA in 5 μl of TE (pH 7.6). Store at −20°C.

$MgCl_2$ (1 M)

Also prepare working solutions of 10, 100, and 250 mM $MgCl_2$.

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***TABLE 8.1** (continued)*

NaCl (5 M)	
NAD	Make a 50 mM stock solution of nicotinamide adenine dinucleotide in water. Store in aliquots at −70°C.
$(NH_4)_2SO_4$ (1 M)	
*Not*I and *Sal*I	These are available from several manufacturers of restriction enzymes.
$Oligo(dT)_{12-18}$	This is sold by Pharmacia. Prepare a stock solution (1 mg/ml in 10 mM Tris · Cl [pH 7.4]). Store in aliquots at −70°C.
Packaging extracts for bacteriophage λ	Packaging extracts are much cheaper to make than to buy. If you plan to construct more than one or two cDNA libraries, it would be worthwhile making a large batch of packaging extract (see Chapter 2, pages 2.98–2.107). However, Gigapack Gold (sold by Stratagene) has a higher efficiency of packaging than most extracts made by occasional users and is recommended for those who make libraries only occasionally.
Phenol:chloroform	Mix equal amounts of phenol and chloroform. Equilibrate the mixture by extracting several times with 0.1 M Tris · Cl (pH 7.6). Store the equilibrated mixture under an equal volume of 0.01 M Tris · Cl (pH 7.6) at 4°C in dark glass bottles.
Poly(A)$^+$ RNA	Approximately 10 μg of poly(A)$^+$ RNA is required to synthesize enough double-stranded cDNA to construct a large library. (One confluent 90-mm plate of cultured mammalian cells yields 1–2 μg of poly(A)$^+$ RNA.) The synthetic reactions will work if less template is available, but losses at each stage will be proportionately higher. Before proceeding, the integrity of the poly(A)$^+$ RNA should be checked by (1) agarose gel electrophoresis (see Chapter 7, page 7.31), (2) translation in a cell-free system followed by SDS-polyacrylamide gel electrophoresis of the resulting polypeptides (see page 8.4), and (3) analysis of the size of the first strand of cDNA synthesized in a pilot reaction (see pages 8.61–8.62).
Reverse transcriptase	In our hands, reverse transcriptase prepared by expression of a gene cloned from a murine retrovirus is better than the enzyme purified from particles of an avian retrovirus. The two enzymes display different pH, salt, and temperature optima, and the reaction conditions given in the experimental protocol on page 8.60 should be used with the murine enzyme only. If the avian enzyme is to be used, prepare the reaction buffer as described on page 8.62. Murine reverse transcriptase expressed from cloned genes is available from a number of manufacturers. Two different clones are currently used to produce the enzyme commercially. The clone used by Pharmacia and Stratagene produces a fusion protein that contains nine amino acids encoded by pBR322 sequences. The other clone was constructed by BRL. The differences between these clones may account for the disparity in units of reverse transcriptase recommended for first-strand synthesis (BRL: 200 units/1–5 μg RNA; Pharmacia: 20–30 units/μg; Stratagene: 200 units/1–5 μg).
RNAase H	This enzyme is purified from *E. coli* by several manufacturers.
RNAase inhibitor	This protein of ~51 kD, purified from human placenta, is a noncompetitive inhibitor of pancreatic RNAase. This inhibitor should be used at a final concentration of 0.5 units/μl of reaction mixture and should be added to the mixture before reverse transcriptase. The inhibitor requires sulfhydryl reagents (1 mM dithiothreitol) for activity and, in fact, denatures irreversibly in the absence of these agents. The inhibitor should be stored at −20°C in glycerol. Do not use preparations of inhibitor that have been frozen and thawed many times; this denatures the protein and releases any bound RNAase that may contaminate the preparation. The protein does not inhibit the activity of RNAase H. It is sold by several manufacturers.

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Sephadex G-50	Add Sephadex G-50 (medium) to distilled sterile water (10 g of dry powder yields 160 ml of slurry). Wash the swollen resin with distilled sterile water several times to remove soluble dextran, which can create problems by precipitating during ethanol precipitation. Finally, equilibrate the resin in TE (pH 7.6), autoclave (10 lb/sq. in. for 15 minutes), and store at room temperature.
Sepharose CL-4B	Wash the paste supplied by the manufacturer several times in sterile TE (pH 7.6) containing 0.1 M NaCl. This washing removes inhibitors of ligation present in some batches of the gel.
Sodium acetate (3 M; pH 5.2)	
Synthetic linkers	Phosphorylated linkers are sold by several manufacturers. If possible, obtain linkers that are at least 4–8 nucleotides longer than the site recognized by the restriction enzyme. Many restriction enzymes inefficiently cleave recognition sites located very close to the ends of DNA molecules.
TE (pH 7.6 and pH 8.0)	0.01 M Tris·Cl (pH 7.6 or pH 8.0), 0.001 M EDTA (pH 8.0).
Trichloroacetic acid (TCA)(10%)	
Tris base (2 M)	
Tris·Cl (2 M; pH 7.4)	
Tris·Cl (1 M; pH 7.6)	
Tris·Cl (2 M; pH 8.0)	
Tris·Cl (1 M; pH 8.3)	This reagent is used to buffer the synthesis of the first strand of cDNA catalyzed by reverse transcriptase purified from avian retroviruses. Since the enzymatic reaction is carried out at 42°C and the pH of Tris solutions is particularly temperature-dependent, the pH of the stock buffer should be adjusted at this temperature. A different buffer is used for murine reverse transcriptase. BRL supplies a 5× reaction buffer for synthesis of the first strand of cDNA when you order murine enzyme.
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside. Make a stock solution by dissolving X-gal in dimethylformamide to make a 20 mg/ml solution. Use a glass or polypropylene tube. The tube containing the solution should be wrapped in aluminum foil to prevent damage by light and should be stored at −20°C. It is not necessary to sterilize X-gal solutions by filtration.

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Protocol for the Synthesis of the First Strand of cDNA

1. Mix in a sterile microfuge tube on ice:

poly(A)$^+$ RNA (1 mg/ml)	10.0 μl
oligo(dT)$_{12-18}$ (1 mg/ml)	10.0 μl
1 M Tris · Cl (pH 7.6)	2.5 μl
1 M KCl	3.5 μl
250 mM $MgCl_2$	2.0 μl
mixture of dNTPs (5 mM each)	10.0 μl
0.1 M dithiothreitol	2.0 μl
RNAase inhibitor	25 units
H_2O to a final volume of 48 μl	

Add 2 μl of murine reverse transcriptase (Pharmacia, 200,000 units/ml; for other manufacturers [see Table 8.1], use an equivalent number of units). Mix well by gentle vortexing. Try to avoid producing bubbles, although this is sometimes difficult because reverse transcriptase is supplied in a buffer containing Triton X-100. If necessary, bubbles may be removed by brief centrifugation in a microfuge.

No radioactive tracer is included in the large-scale first-strand reaction described above. To analyze the material produced in the reaction, it is necessary to carry out a small-scale parallel reaction:

a. After all components of the large-scale reaction have been mixed at 4°C, place 2.5 μl of the reaction in a fresh, small (0.5-ml) microfuge tube.

b. Add 0.1 μl of [α-^{32}P]dCTP (400 Ci/mmole, 10 μCi/μl) to the small-scale reaction.

c. Incubate both reactions for 1 hour at 37°C.

d. Count the total and TCA-precipitable radioactivities in 0.5 μl of the small-scale reaction. Store the large-scale reaction at 4°C.

e. Store the remainder of the small-scale reaction at −20°C for later analysis by electrophoresis through an alkaline agarose gel (see Chapter 6, pages 6.20–6.21).

If you are using avian reverse transcriptase, the buffer should be altered appropriately (see Note iii).

2. Calculate the amount of first strand synthesized as follows:

a. Since 10 μl of a solution containing all four dNTPs at a concentration of 5 mM each was used (i.e., 10 μl of 20 mmoles/liter of total dNTP), the large-scale first-strand reaction therefore contains

$$20 \text{ nmoles}/\mu\text{l dNTP} \times 10\ \mu\text{l} = 200 \text{ nmoles of dNTP}$$

b. Because the molecular weight of each dNTP incorporated into DNA is approximately 330, the reaction is capable of generating a total of

$$200 \text{ nmoles} \times 330 \text{ ng/nmole} = 66\ \mu\text{g of DNA}$$

c. Therefore, from the results of the small-scale reaction,

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$$\frac{\text{cpm incorporated}}{\text{total cpm}} \times 66\ \mu g = \mu g \text{ of first strand of cDNA synthesized}$$

The yield of first strand is usually close to 50% of the weight of the poly(A)$^+$ RNA in the reaction mixture.

Notes

i. An alternative procedure to monitor synthesis of the first strand of cDNA is to use a small amount of [α-^{32}P]dCTP (10 μCi total) in the large-scale reaction described above and a much greater amount (100 μCi total) of radiolabeled dNTP in the subsequent reaction to synthesize the second strand of cDNA. This procedure is less desirable because it is then necessary to expose the alkaline agarose gel to X-ray film for long periods of time to obtain a suitable image of the first-strand products.

ii. If enough poly(A)$^+$ RNA is available, it is advisable to determine empirically the amount of reverse transcriptase that results in the highest yields and longest cDNAs. Small-scale reactions (each containing 0.5–1.0 μg of poly(A)$^+$ RNA) should be set up using the conditions described below and including varying amounts of reverse transcriptase (10–200 units/μg of RNA). The yield of cDNA should be determined by precipitation with ice-cold TCA (see Appendix E), and its size should be determined by electrophoresis through alkaline agarose gels (see Chapter 6, pages 6.20–6.21). For large-scale synthesis of cDNA, use the concentration of enzyme that gives the greatest yield of long cDNA. If limiting amounts of poly(A)$^+$ RNA are available, use 40–50 units of reverse transcriptase per microgram of RNA.

Pilot reactions for synthesis of the first strand of cDNA

a. Mix one or more pilot reactions on ice in sterile microfuge tubes:

poly(A)$^+$ RNA (0.5–1.0 mg/ml)	1.0 μl
oligo(dT)$_{12-18}$ (1 mg/ml)	2.0 μl
1 M Tris · Cl (pH 7.6)	1.5 μl
1 M KCl	1.5 μl
250 mM $MgCl_2$	1.0 μl
mixture of dNTPs (5 mM each)	5.0 μl
0.1 M dithiothreitol	1.0 μl
RNAase inhibitor	10 units
[α-^{32}P]dCTP (400 Ci/mmole; 10 μCi/μl)	1.0 μl
H_2O to a final volume of 25 μl	

Add to each assay varying amounts of murine reverse transcriptase (10–200 units) per microgram of mRNA. Mix well by gentle vortexing. Remove bubbles by brief centrifugation in a microfuge.

Incubate for 2 hours at 37°C.

If you are using avian reverse transcriptase, the buffer for the reaction should be altered appropriately (see Note iii).

b. Count the total and TCA-precipitable radioactivities in 2.5 μl of the reaction. Store the remainder of the reaction at −20°C for later analysis by electrophoresis through an alkaline agarose gel (see Chapter 6, pages 6.20–6.21).

c. Calculate the amount of first strand synthesized as follows:

$$\frac{\text{cpm incorporated}}{\text{total cpm}} \times 33\ \mu g = \mu g \text{ of first strand of cDNA synthesized}$$

iii. A reaction mixture buffered at pH 8.3 is generally used to synthesize cDNA with reverse transcriptase purified from particles of avian retroviruses:

poly(A)$^+$ RNA (1 mg/ml)	10.0 μl
oligo(dT)$_{12-18}$ (1 mg/ml)	10.0 μl
1 M Tris · Cl (pH 8.3) at 42°C	2.5 μl
1 M KCl	7.0 μl
250 mM $MgCl_2$	2.0 μl
mixture of dNTPs (5 mM each)	10.0 μl
0.1 M dithiothreitol	2.0 μl
RNAase inhibitor	25 units
H_2O to a final volume of 48 μl	

Add 40 units of avian reverse transcriptase and mix as described above. Incubate for 2 hours at 42°C.

Preparations of reverse transcriptase purified from particles of avian retroviruses usually contain between 5 and 20 units/μl. If you need to add more than 4 μl of this enzyme, you will alter the pH and ionic strength of the reaction mixture (avian reverse transcriptase is usually supplied in a storage buffer containing 200 mM potassium phosphate [pH 7.2]). In this case, it might be advisable to increase the volume of the reaction mixture or to reduce the amount of poly(A)$^+$ RNA and enzyme. This problem does not occur with murine reverse transcriptase purified from *E. coli* expressing the enzyme from a cloned copy of the gene; this enzyme is supplied in Tris buffer.

iv. Preparations of reverse transcriptase (particularly those purified from particles of avian retroviruses) are often contaminated with RNAase. This problem can be alleviated by including a potent protein inhibitor of RNAase in the first-strand synthesis reaction (de Martynoff et al. 1980). This inhibitor, which is purified from human placenta, should be used at a final concentration of 0.5 units/μl of reaction mixture and should be added to the mixture before reverse transcriptase. The inhibitor requires sulfhydryl reagents (1 mM dithiothreitol) for activity and, in fact, denatures irreversibly in the absence of these agents. The inhibitor should be stored at −20°C in glycerol. Do not use preparations of inhibitor that have been frozen and thawed many times; this denatures the protein and releases any bound RNAase that may contaminate the preparation.

v. Many workers have used methylmercuric hydroxide to denature the poly(A)$^+$ RNA before synthesis of the first strand of cDNA. This procedure is believed to alleviate potential problems caused by secondary structure in mRNA. However, in our hands, there is no noticeable effect

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of denaturation on the size of the bulk cDNA, and furthermore, methylmercuric hydroxide is very toxic and nasty to handle. Unless you have good reason to believe that your particular mRNA has a greater-than-average amount of secondary structure, there seems to be little reason to use methylmercuric hydroxide. However, because of the lower temperature used for synthesis with the murine reverse transcriptase (37°C), as compared with that used with the avian enzyme (42°C), this prior denaturation step may be more important when using the murine enzyme.

Caution: Methylmercuric hydroxide is extremely toxic. It is also volatile. Therefore, all manipulations of solutions containing concentrations of methylmercuric hydroxide in excess of 10^{-2} M should be carried out in a chemical hood. Wear gloves when handling such solutions. All solid and liquid wastes should be treated as toxic materials and disposed of accordingly.

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Protocol for the Synthesis of the Second Strand of cDNA

The unlabeled first-strand reaction needs no further treatment before proceeding to the synthesis of the second strand of cDNA.

1. Add the following reagents directly to the first-strand reaction mixture:

10 mM $MgCl_2$	70.0 μl
2 M Tris · Cl (pH 7.4)	5.0 μl
[α-^{32}P]dCTP (400 Ci/mmole; 10 μCi/μl)	10.0 μl
1 M $(NH_4)_2SO_4$	1.5 μl
RNAase H (1000 units/ml)	1.0 μl
E. coli DNA polymerase I (10,000 units/ml)	4.5 μl

Incubate for 4 hours at 16°C.

2. Add:

50 mM NAD	1.0 μl
E. coli DNA ligase (100,000 units/ml)	1.0 μl
bacteriophage T4 polynucleotide kinase (3000 units/ml)	1.0 μl

Incubate for 15 minutes at room temperature. *E. coli* DNA ligase will repair nicks in the DNA and the kinase will phosphorylate the 5′-hydroxyl group of the oligo(dT) primers.

3. Add 5 μl of 0.5 M EDTA (pH 8.0) to stop the reaction. Remove a small aliquot (3 μl) and store the remainder of the sample at −20°C until needed in step 6. Count the total and TCA-precipitable radioactivities in 0.5 μl of the small aliquot.

4. Calculate the weight of the cDNA synthesized in the second-strand reaction, taking into account the amount of dNTPs already incorporated into the first strand of cDNA:

$$\frac{\text{cpm incorporated in the second-strand reaction}}{\text{total cpm}} \times (66\ \mu g - x\ \mu g)$$

$$= \mu g \text{ of the second strand of cDNA synthesized}$$

where x = weight of the first strand of cDNA. The amount of second-strand cDNA synthesized is usually 70–80% of the weight of the first strand.

5. Analyze the sizes of the radiolabeled first- and second-strand products by electrophoresis through an alkaline 1% agarose gel (see Chapter 6, pages 6.20–6.21), using end-labeled fragments of a *Hin*dIII digest of wild-type bacteriophage λ DNA as markers (see Chapter 10). If the reactions have worked well, the first strand of cDNA should exhibit a broad distribution of sizes (300 bases to more than 5 kb), with the majority of the material ranging between 1 and 2 kb.

It is very important that the size of the second strand of cDNA be identical to that of the first. A detectable increase in size indicates that a significant proportion of the

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second-strand molecules have been generated by self-priming rather than by replacement synthesis. This can be verified as follows:

a. Treat two aliquots of radiolabeled first strand and two aliquots of radiolabeled second strand with 1 mM EDTA (pH 8.0), 300 mN NaOH for 30 minutes at 60°C. This hydrolyzes the RNA and denatures the DNA in the samples. Each aliquot should contain at least 10^4 cpm.
b. Add 0.1 volume of 3 N HCl, 0.1 M Tris·Cl (pH 7.4) to each aliquot.
c. Purify the DNAs by extraction with phenol:chloroform and precipitation with ethanol.
d. Redissolve the DNAs in 10 μl of H_2O. Add 1 μl of 3 M NaCl. Incubate the DNAs for 5 minutes at 68°C to allow "hairpins" to form in the DNAs.
e. Add 20 μl of H_2O and 3 μl of 10× nuclease-S1 buffer.

10× Nuclease-S1 buffer

2 M NaCl
0.5 M sodium acetate (pH 4.5)
10 mM $ZnSO_4$
5% glycerol

f. Add 5 units of nuclease S1 to one of the two tubes containing the first strand of cDNA and to one containing the second strand of cDNA. Incubate all four tubes for 30 minutes at 37°C.
g. Analyze the size of first- and second-strand products by electrophoresis through an alkaline 1% agarose gel (see Chapter 6, pages 6.20–6.21), using end-labeled fragments of a *Hin*dIII digest of wild-type bacteriophage λ DNA as markers (see Chapter 10).

Note: The first-strand molecules should be completely hydrolyzed by nuclease S1, and no more than 10–15% of the second-strand molecules should be resistant to digestion with the enzyme. If the proportion of self-primed molecules is unacceptably high, it is often helpful to adjust the amount of RNAase H used during the synthesis of the second strand. In addition, self-priming may be suppressed by including actinomycin D (50 μg/ml) or 4 mM sodium pyrophosphate (Rhyner et al. 1986) in first-strand reactions catalyzed by avian reverse transcriptase. Sodium pyrophosphate inhibits the activity of the murine enzyme (Roth et al. 1985).

Caution: Actinomycin D is a teratogen and carcinogen. Stock solutions should be prepared, wearing gloves and a mask, in a chemical hood, not on an open bench.

6. Thaw the remainder of the sample (stored in step 3) and extract with an equal volume of phenol:chloroform. Separate the unincorporated dNTPs from the cDNA by spun-column chromatography through Sephadex G-50 equilibrated in TE (pH 7.6) containing 10 mM NaCl (see Appendix E).

7. Precipitate the eluted cDNA by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Store the sample at room temperature for at least 15 minutes. Recover the precipitated DNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Use a hand-held minimonitor to check that all of the radioactivity has been precipitated. Wash the pellet with 70% ethanol and recentrifuge. Gently aspirate all of the fluid (check to see that none of the radioactivity is in the aspirated fluid), and allow the pellet to dry in the air.

8. Redissolve the DNA in 80 μl of TE (pH 7.6) if it is to be methylated by M.*Eco*RI methylase (see below) or in 29 μl of TE (pH 7.6) if it is to be ligated directly to *Not*I or *Sal*I linkers (see page 8.68).

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Methylation of cDNA

1. Add to the resuspended DNA:

2 M Tris · Cl (pH 8.0)	5 μl
5 M NaCl	2 μl
0.5 M EDTA (pH 8.0)	2 μl
20 mM *S*-adenosyl-L-methionine	1 μl
H_2O to a final volume of 96 μl	

M.*Eco*RI methylase is inhibited by Mg^{++} ions.

2. Remove two aliquots (2 μl each) and place in small (0.5-ml) microfuge tubes. Number these aliquots 1 and 2. Store at 0°C.

It is important to use small microfuge tubes to minimize evaporation of the sample during subsequent incubation.

3. Add 2 μl of M.*Eco*RI methylase (80,000 units/ml) to the large-scale reaction and mix at 0°C.

4. Remove two additional aliquots (2 μl each) from the large-scale reaction and place in small microfuge tubes. Number these aliquots 3 and 4.

5. To all four aliquots, add 100 ng of a plasmid such as Xf3 or pBR322 that has been digested to completion with *Pst*I, purified by extraction with phenol:chloroform and precipitation with ethanol, and dissolved in TE (pH 8.0) at a concentration of 100 ng/μl. Incubate all four control reactions and the large-scale reaction for 1 hour at 37°C.

6. Stop the reactions by heating to 68°C for 15 minutes. Extract the bulk cDNA reaction once with phenol:chloroform and once with chloroform. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Mix well and store at −20°C until the results of the control reactions are available.

7. Analyze the four control reactions as follows:

   a. Add to each control reaction:

0.1 M $MgCl_2$	2 μl
10× *Eco*RI buffer	2 μl
H_2O to 20 μl	

   b. Add 20 units of *Eco*RI to reactions 2 and 4.

   c. Incubate all four samples for 1 hour at 37°C, and analyze them by electrophoresis through a 1% agarose gel.

Often, the cDNA can be seen as a background smear in the agarose gel. Its molecular weight should not be affected by digestion with *Eco*RI after methylation. The unmethylated plasmid DNA should be cleaved into two fragments by *Eco*RI, whereas the methylated DNA should be protected from the enzyme. If this is the case, proceed to step 8; if not, repeat the methylation reaction.

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8. Recover the precipitated cDNA (step 6) by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Add 200 μl of 70% ethanol and recentrifuge. Use a hand-held minimonitor to check that all of the radioactivity is recovered in the pellet. Remove the ethanol by gentle aspiration, dry the pellet in the air, and then redissolve the DNA in 29 μl of TE (pH 7.6).

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Ligation to Synthetic Phosphorylated Linkers

1. Heat the sample to 68°C for 5 minutes to denature any double-stranded structures that may have formed from protruding single-stranded termini of the cDNA molecules. Cool to 37°C.

2. Add:

5 × bacteriophage T4 DNA polymerase repair buffer	10 μl
mixture of dNTPs (5 mM each)	5 μl
H_2O to a final volume of 50 μl	

5 × Bacteriophage T4 DNA polymerase repair buffer

90 mM $(NH_4)_2SO_4$
0.33 M Tris · Cl (pH 8.3)
33 mM $MgCl_2$
50 mM β-mercaptoethanol

This buffer should be stored in small aliquots at −20°C.

Add 1–2 units of bacteriophage T4 DNA polymerase (500 units/ml) and incubate for 15 minutes at 37°C.

3. Stop the reaction by adding 1 μl of 0.5 M EDTA (pH 8.0).

4. Extract the sample with phenol:chloroform, and remove the unincorporated dNTPs by spun-column chromatography through Sephadex G-50 (see Appendix E).

5. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. Store the sample at 4°C for at least 15 minutes. Recover the precipitated cDNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Dry the pellet in the air, and then redissolve it in 13 μl of 10 mM Tris · Cl (pH 7.6).

6. Add:

10 × ligation buffer	2 μl
phosphorylated linkers (800–1000 ng)	2 μl
bacteriophage T4 DNA ligase (100,000 Weiss units/ml)	1 μl
10 mM ATP	2 μl

Mix and then incubate for 8–12 hours at 16°C.

It is essential to use a vast molar excess of linkers (> 100-fold) to ensure that the ends of the cDNA become ligated to a linker and not to each other.

For definition of Weiss unit, see Table 8.1.

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10 × Ligation buffer

0.5 M Tris · Cl (pH 7.6)
100 mM $MgCl_2$
100 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

This buffer should be stored in small aliquots at −20°C.

7. Withdraw 0.5 μl from the reaction and store at 4°C. Inactivate the ligase in the remainder of the reaction by heating at 68°C for 15 minutes.
   Add:

10 × restriction enzyme buffer	20 μl
H_2O	150 μl
restriction enzyme	200 units

   Mix at 0°C. As a control, transfer an aliquot (2 μl) of the reaction to a small (0.5-ml) microfuge tube. To the 2-μl aliquot, add 100 ng (in a volume of no more than 0.5 μl) of a linearized plasmid that contains an internal site for the particular restriction enzyme used.

8. Incubate the large-scale reaction and the control for 2 hours at 37°C. Analyze the DNA in the control sample and the 0.5-μl aliquot withdrawn at step 7 by electrophoresis through a 1% agarose gel, using as markers plasmid DNA that has been digested to completion with (a) *Pst*I and (b) a combination of *Pst*I and the particular restriction enzyme used.
   If the ligation has worked well, the ligated linkers should be visible as a smear at the bottom of the gel. If digestion with the restriction enzyme is complete, the smear of linkers should disappear and the plasmid DNA in the control sample should be cleaved into two fragments.

9. Purify the cDNA by extraction with phenol:chloroform and precipitation with ethanol as described in steps 4 and 5. Redissolve the cDNA in 20 μl of TE (pH 7.6). Add 2.5 μl of a solution of 0.25% bromophenol blue, 50% glycerol in water. This simplifies the subsequent step of loading the cDNA onto a column of Sepharose CL-4B.

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Size Selection of cDNA

The digested cDNA is fractionated by chromatography through a column of Sepharose CL-4B to remove the remnants of the digested linkers and to eliminate cDNA molecules smaller than 500 bp in length. A long, narrow column (27 cm × 0.3 cm) is needed to achieve adequate separation from the large molar excess of digested linkers.

1. Prepare a column (27 cm × 0.3 cm) of Sepharose CL-4B extensively washed in TE (pH 7.6) containing 0.1 M NaCl:

   a. Using a hypodermic needle with a bent end, pull the cotton wool pledget halfway out of the end of a sterile, disposable 1-ml pipette (e.g., Kimax 720120; Baxter). Cut the pledget in half with sterile scissors. Discard the loose piece of cotton wool. Using filtered compressed air, blow the remainder of the pledget to the narrow end of the pipette.

   b. Attach a piece of sterile polyvinyl chloride tubing (of the type normally used in peristaltic pumps) to the narrow end of the pipette. Dip the wide end into a solution of TE (pH 7.6) containing 0.1 M NaCl in a beaker. Attach the tubing to an Erlenmeyer flask connected to a vacuum line. Apply gentle suction until the pipette is filled with buffer. Close the tubing with a hemostat.

   c. Attach a piece of vinyl bubble tubing (e.g., Fisher 14-170-13A) to the wide end of the pipette. Fill the bubble tubing with Sepharose CL-4B equilibrated with TE (pH 7.6) containing 0.1 M NaCl (see Appendix E). Allow the slurry to settle for a few minutes and then release the hemostat. The column will form as the buffer drips from the pipette. If necessary, add more Sepharose CL-4B until the packed matrix almost fills the pipette. Wash the column with several column volumes of TE (pH 7.6) containing 0.1 M NaCl.

2. Close the tubing at the bottom of the column with a hemostat. Using a pasteur pipette, remove the fluid above the Sepharose CL-4B. Apply the cDNA to the column (in a volume of 50 μl or less). Release the hemostat and allow the cDNA to enter the gel matrix. Wash the microfuge tube used to store the cDNA with 50 μl of TE (pH 7.6), and apply this to the column. Fill the bubble tubing with TE (pH 7.6) containing 0.1 M NaCl.
   Important: Do not allow the column to run dry at any stage!

3. Monitor the progress of the cDNA through the column using a hand-held minimonitor. Begin collecting 2-drop fractions (60 μl) in microfuge tubes just as the radioactive cDNA reaches the bottom of the matrix. Continue collecting fractions until all of the radioactivity has eluted from the column.

4. Measure the radioactivity in each fraction by Cerenkov counting (see Appendix E). When plotted, the data should look like Figure 8.15.

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5. Analyze small aliquots of each fraction (~ 5 μl) by electrophoresis through a 1% agarose gel, using as markers end-labeled fragments of DNA of known size (200 bp–5 kb). (Cast the agarose gel as thin as possible to speed up the drying process later.) The amount of radioactivity in the markers should be approximately 0.3 times the amount in the peak fractions of cDNA. Store the remainder of the fractions at −20°C until the autoradiograph of the agarose gel is available.

   After electrophoresis, transfer the gel to a piece of Whatman 3MM paper, cover it with Saran Wrap, and dry it on a commercial gel dryer. Heat the gel to 50°C for the first 20–30 minutes of drying and then turn off the heat. Continue drying the gel under vacuum for a further 1–2 hours. Expose the dried gel to X-ray film at −70°C with an intensifying screen (see Appendix E). The autoradiograph should be similar to that shown in Figure 8.16.

6. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol to fractions containing cDNA molecules no shorter than 500 bp in length. Be very conservative in selecting these fractions. Do not include any fractions that contain any traces of cDNAs smaller than 500 bp; otherwise, the library will contain a preponderance of short clones. Store the samples for at least 15 minutes at 4°C. Recover the DNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge.

7. Redissolve the DNA in a total volume of 20 μl of 10 mM Tris · Cl (pH 7.6). Determine the amount of radioactivity present in a small aliquot, and calculate the total amount of cpm available in the selected fractions. Calculate the total quantity of cDNA available for ligation to bacteriophage λ arms (see page 8.64 for calculation of second-strand cDNA data):

$$\frac{\text{cpm available}}{\text{cpm incorporated into second strand}} \times 2 \times \mu\text{g second-strand cDNA synthesized} = \mu\text{g of cDNA available for ligation}$$

   If everything has gone well, 10 μg of poly(A)$^+$ RNA should yield 250–400 ng of cDNA whose size is larger than 500 bp in length.

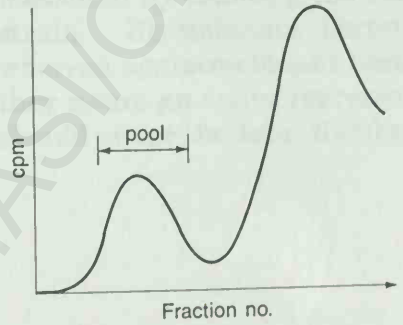

FIGURE 8.15

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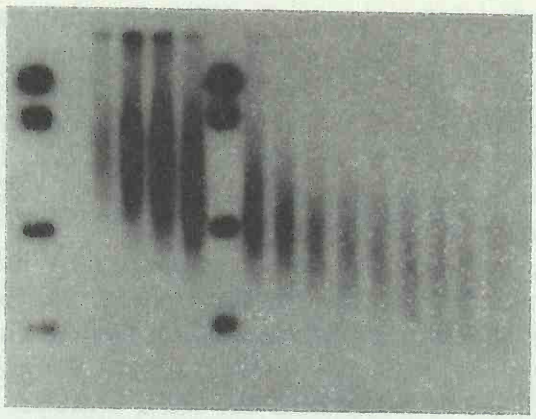

FIGURE 8.16
Size fractionation of cDNA by chromatography through Sepharose CL-4B.

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Ligation to Bacteriophage λ Arms

Because of the difficulties involved in determining the molar concentration of the termini of cDNA that can be ligated to bacteriophage λ arms, it is necessary to carry out a series of trial ligations and packaging reactions. In these reactions, a constant amount of bacteriophage λ arms is ligated to varying amounts of cDNA. The aim is to determine the amount of cDNA that yields at least 5×10^6 recombinant bacteriophages (i.e., a mammalian cDNA library of reasonable size).

The ligation reactions need to be carefully arranged so as to minimize the chance that a single recombinant bacteriophage will contain more than one cDNA molecule. This is achieved by using a molar ratio of phosphorylated arms to cDNA such that only 5% of the resulting bacteriophages are recombinant. If dephosphorylated arms are used, the background of nonrecombinant bacteriophages is effectively suppressed, and it is therefore impossible to determine the amount of cDNA required to yield a population of bacteriophages that contains 5% recombinants. In this case, the cDNA is titrated to determine the amount that yields a tenfold stimulation of total plaques over the background of vector alone.

The use of dephosphorylated arms or arms with noncompatible termini produced by partial filling is strongly recommended when constructing cDNA libraries in bacteriophages such as λgt11, λgt18–23, and λZAP, which have no system that allows selection against parental bacteriophages. The number of nonrecombinants can be reduced by approximately 100-fold by using dephosphorylated arms. In this system, nonrecombinant bacteriophages form blue plaques on strains of *E. coli* such as Y1090*hsdR* in the presence of the inducer IPTG and the chromogenic substrate X-gal; recombinant plaques are colorless.

Nonrecombinant bacteriophages do not present a problem when bacteriophages such as λgt10 are used as vectors, since a single round of growth on *E. coli* strains carrying an *hfl*$^-$ mutation eliminates the vast majority of nonrecombinants from the population. Recombinants form clear plaques on the *E. coli hfl*$^-$ strain BNN102 (because insertion of the cDNA inactivates the *c*I gene, which codes for repressor). Nonrecombinant bacteriophages establish repression so effectively that their efficiency of plating is greatly reduced. The absolute number of cDNA recombinants constructed in λgt10 can therefore be obtained by counting the number of plaques formed on BNN102. The proportion of recombinants in the bacteriophage stock can be measured by counting the ratio of clear to turbid plaques on C600, an *hfl*$^+$ strain. Recombinant bacteriophages form clear plaques on this strain, whereas nonrecombinant bacteriophages give rise to turbid plaques (because they retain an active repressor). The number of clear plaques on strain C600 should equal the total number of plaques on strain BNN102.

1. Set up four test ligation/packaging reactions as follows:

	A	B	C	D
Bacteriophage λ (0.5 μg/ml)	1.0 μl	1.0 μl	1.0 μl	1.0 μl
10× Ligation buffer	1.0 μl	1.0 μl	1.0 μl	1.0 μl
cDNA	0 ng	3 ng	10 ng	25 ng
Bacteriophage T4 DNA ligase (100,000 Weiss units/ml)	0.1 μl	0.1 μl	0.1 μl	0.1 μl
10 mM ATP	1.0 μl	1.0 μl	1.0 μl	1.0 μl
H_2O to a final volume of	10 μl	10 μl	10 μl	10 μl

For definition of Weiss unit, see Table 8.1.

10 × Ligation buffer

0.5 M Tris · Cl (pH 7.6)
100 mM $MgCl_2$
100 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

This buffer should be stored in small aliquots at −20°C

Incubate the ligation mixtures for 4 hours at 16°C. Store the unused portion of the cDNA at −20°C.

Package 5 μl of each ligation into bacteriophage λ particles (see Chapter 2, page 2.104). Because the size of the final cDNA library depends on the efficiency with which recombinant bacteriophage λ genomes are packaged into infectious bacteriophage particles, it is essential to use packaging mixtures of high potency that yield $>5 \times 10^8$ pfu/μg of bacteriophage λ DNA.

After the packaging reaction is complete, add 0.5 ml of SM to each mixture.

2. Have available freshly prepared overnight cultures of the appropriate strains of *E. coli*. Plate 10 μl and 100 μl of a 10^{-2} dilution of each packaging mixture on each strain (see below). Incubate the plates for 8–12 hours at 37°C or 42°C.

Plaque formation with λgt10: Grow *E. coli* strains C600 and BNN102 in NZCYM medium containing 0.2% maltose. (BNN102 grows much more slowly than C600.) Prepare stocks of plating bacteria of both strains as described in Chapter 2, page 2.60. (It may be necessary to suspend the bacteria of strain BNN102 in a smaller volume of 10 mM $MgSO_4$ than usual to attain a density of $\sim 1.5 \times 10^9$ cells/ml.)

Measure the number of infectious bacteriophage particles in the packaging mixtures as described in Chapter 2, page 2.61, using LB agar plates and LB top agarose. It is easier to distinguish between clear and turbid plaques on relatively sparse bacterial lawns. Plaques of recombinant λgt10 bacteriophages appear on lawns of C600 cells after incubating for 6–7 hours at 37°C; plaques on BNN102 take 1–2 hours longer to appear.

Plaque formation with λgt11 and λgt18–23: Grow *E. coli* strain Y1090*hsdR* in NZCYM medium containing 0.2% maltose. Prepare a stock of plating bacteria in 10 mM $MgSO_4$ as described in Chapter 2, page 2.60.

Measure the number of infectious bacteriophage particles in the packaging mixtures as described on page 2.61, using 3 ml of LB top agarose containing 40 μl of a stock

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solution of X-gal (20 mg/ml in dimethylformamide) and 4 μl of a solution of IPTG (200 mg/ml) for each 90-mm petri dish. Incubate the plates for 8–10 hours at 42°C.

Plaque formation with λZAP: Grow *E. coli* strain BB4 in NZCYM medium containing 0.2% maltose. Prepare a stock of plating bacteria in 10 mM $MgSO_4$ as described in Chapter 2, page 2.60.

Measure the number of infectious bacteriophage particles in the packaging mixtures as described on page 2.61, using 3 ml of LB top agarose containing 40 μl of a stock solution of X-gal (20 mg/ml in dimethylformamide) and 4 μl of a solution of IPTG (200 mg/ml) for each 90-mm petri dish. Incubate the plates for 8–10 hours at 42°C.

Note: Y1090*hsdR* should not be used with the λZAP vectors, since this strain contains a plasmid (pMC9) that is homologous to the phagemid sequences within the λZAP vector. This homology could promote recombination between the bacteriophage λ vector and the plasmid.

Plaque formation with λZAPII: Grow *E. coli* strain XL1-Blue in NZCYM medium containing 0.2% maltose. Prepare a stock of plating bacteria in 10 mM $MgSO_4$ as described in Chapter 2, page 2.60.

Measure the number of infectious bacteriophage particles in the packaging mixtures as described on page 2.61, using 3 ml of LB top agarose containing 40 μl of a stock solution of X-gal (20 mg/ml in dimethylformamide) and 4 μl of a solution of IPTG (200 mg/ml) for each 90-mm petri dish. Incubate the plates for 8–10 hours at 42°C.

Note: Y1090*hsdR* should not be used with the λZAP vectors, since this strain contains a plasmid (pMC9) that is homologous to the phagemid sequences within the λZAP vector. This homology could promote recombination between the bacteriophage λ vector and the plasmid.

3. Count the number of recombinant and nonrecombinant plaques. Ligation A should yield no recombinant plaques, whereas ligations B, C, and D should yield increasing numbers of recombinant plaques. From the number of recombinant plaques, calculate the efficiency of cloning of cDNA (plaque-forming units per nanogram of cDNA). If everything has gone well, the efficiency should be at least 2×10^4 pfu per nanogram of cDNA. The total yield of recombinants from 10 μg of poly(A)$^+$ RNA should be in excess of 5×10^6.

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Analysis of cDNA Inserts

1. Pick 12 recombinant bacteriophage plaques, grow small-scale lysates, and prepare DNAs for digestion with the appropriate restriction enzyme as described in Chapter 2.

2. Analyze the size of the cDNA inserts by electrophoresis through a 1% agarose gel, using as markers fragments of DNA 500 bp to 5 kb in length. If the recombinant bacteriophages contain inserts of different sizes and if the average size of the inserts is approximately 1 kb or greater, it is worthwhile proceeding to the next stage (i.e., to generate a complete cDNA library). If the average size of the inserts is significantly less than 1 kb, then the quality of the library is not as high as it could be. Go back and reanalyze the quality of the starting mRNA and the cDNA reactions, paying special attention to the precautions listed on pages 8.54–8.55.

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1. If you are using dephosphorylated arms, calculate the amount of cDNA that results in a tenfold increase over background in the number of nonrecombinant plaques. If you are using phosphorylated arms, calculate the amount of cDNA that results in a population of bacteriophages consisting of approximately 5% recombinants. Using the test ligations as a guide, set up a large-scale ligation reaction, increasing the amounts of all components proportionately. Be sure to set up a control reaction that contains only bacteriophage λ arms and no cDNA. Package all of the ligation mixture by setting up a series of packaging reactions, each of which contains 0.5 μg of bacteriophage λ arms (see Chapter 2, page 2.104). It is important that the ratio of ligation reaction to packaging mixture be identical to that used in the trial packaging reactions.

2. Pool the packaged particles and determine the titer of the resulting bacteriophage stock on the appropriate bacterial strain (see Chapter 2, page 2.61).

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AMPLIFICATION OF LIBRARIES CONSTRUCTED IN BACTERIOPHAGE λgt10: SELECTION AGAINST PARENTAL BACTERIOPHAGES

To establish a permanent supply of the library, it should be amplified by growth on *E. coli* strain BNN102 on agar plates as described below. If the library will be screened only once, this amplification step can be omitted and the bacteriophages can be plated directly on strain BNN102 at a density appropriate for screening (see Chapter 2, page 2.61).

1. For amplification, add 10^5 bacteriophages or 200 μl of the packaging mixture (whichever is the smaller volume) to 300 μl of plating bacteria of strain BNN102. Incubate for 20 minutes at 37°C. Then plate on a petri dish containing NZCYM agar, and incubate for 8–10 hours at 37°C.

   In some cases, the use of large volumes of packaging mixture leads to a reduction in plating efficiency. This should be checked before amplification by a reconstruction experiment in which known numbers of bacteriophage λgt10 (e.g., 1000 pfu) are mixed with increasing amounts of packaging mixture and plated on *E. coli* strains C600 and BNN102.

2. Overlay the plates with 15 ml of SM for 2 hours at room temperature or overnight at 4°C.

3. Harvest the SM carefully, and remove cellular debris by centrifugation at 7000*g* for 30 minutes at 4°C. The cDNA library will be stable for several months when stored at 4°C in small (1-ml) aliquots in tightly closed tubes, each of which contains 20–30 μl of chloroform. For longer-term storage, add dimethyl sulfoxide to a final concentration of 7% and store aliquots at −70°C.

4. Determine the titer of the library on *E. coli* strains C600 and BNN102 (see Chapter 2, page 2.61). The titer of clear plaques on strain C600 should be 10^{10}–10^{11} pfu/ml and the ratio of clear to turbid plaques should be greater than 20:1.

   The size of the library is determined by the number of independent recombinant plaques that are amplified. For example, if ten plates, each containing 100,000 plaques, are used for amplification, the resulting cDNA library will have 1,000,000 members. The titer of the library has nothing to do with its size.

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cDNA libraries constructed in expression vectors such as λgt11, λgt18, λgt20, and λgt22 should be amplified on *E. coli* strain Y1090*hsdR*. cDNA libraries constructed in λZAP vectors should be amplified on *E. coli* strain BB4 (or strain XL1-Blue for λZAPII). These strains are defective for host-controlled restriction but carry an active methylation system. Potential sites for restriction by the *Eco*K system will become methylated during amplification so that, if necessary, the recombinants can be used to infect a restriction-competent strain of *E. coli*. During amplification, it is important to suppress the production of potentially toxic proteins by the recombinant bacteriophages. Strain Y1090*hsdR* carries a plasmid (pMC9: pBR322 *lacI*^{q}) that expresses the *lac* repressor and prevents synthesis of fusion proteins controlled by the β-galactosidase promoter. λZAP cannot be grown on strain Y1090*hsdR* due to the homology between the plasmid pMC9 and phagemid sequences within the λZAP vector.

1. Mix 10^5 bacteriophages with 600 μl of plating bacteria of *E. coli* strain Y1090*hsdR* (or strain BB4 or XL1-Blue for λZAP or λZAPII, respectively). After incubating for 20 minutes at 37°C, plate the infected culture on a 150-mm petri dish containing NZCYM agar. Incubate for 12 hours at 42°C (to prevent the formation of lysogens).

2. Prepare and store the library as described in steps 2–3 on page 8.78.

3. Titrate the λgt library on strain Y1090*hsdR* (or the λZAP or λZAPII library on strain BB4 or XL1-Blue, respectively) on LB agar plates using 3 ml of top agarose containing 40 μl of a stock solution of X-gal (20 mg/ml in dimethylformamide) and 4 μl of a solution of IPTG (200 mg/ml). The ratio of blue to colorless plaques is a measure of the proportion of the library that consists of nonrecombinant bacteriophages.

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PROBLEMS COMMONLY ENCOUNTERED WITH cDNA CLONING

Problems commonly encountered in cloning cDNA include:

- *The first strand of cDNA is too short or the yield is too low.* Although these problems can result from inadequacy in any of the components (enzyme, buffer, or substrates) used for synthesis of the first strand of cDNA, they far more frequently reflect the poor quality of the mRNA used as template. Recheck the integrity of the mRNA preparation by the tests described on pages 8.4–8.5. If the problem still persists, check the other components of the first-strand reaction, using an mRNA template whose integrity can be checked before and after the reaction. Globin mRNA purified from reticulocytes and IgG mRNA purified from lymphocytes, myelomas, or hybridomas are commonly used for this purpose. Alternatively, existing cDNAs cloned in an Okayama-Berg vector can be subcloned into a plasmid such as Bluescript (see Chapter 1, page 1.20) and used as templates for the synthesis in vitro of an RNA that carries a poly(A) tract very close to its 3′ terminus. Such RNAs serve as extremely good templates for oligo(dT)-primed synthesis of the first strand of cDNA.

  Note: cDNAs cloned in Okayama-Berg vectors are better suited for this purpose than other cDNAs, since they generally contain longer tracts of poly(A).

- *The library contains shorter inserts of cDNA than desired.* This problem generally has one of the following causes:

  1. Inefficiency in the synthesis of the second strand of cDNA, resulting in double-stranded molecules that contain gaps or traces of mRNA—a possibility that can be eliminated if the size of the labeled second strand of cDNA is adequate as determined by electrophoresis through an alkaline 1% agarose gel (see Chapter 6, pages 6.20–6.21).

  2. Small pieces of DNA (fragments of cDNA or undigested oligomers of linkers) contaminate the final preparation of cDNA. The best course of action is to redigest the cDNA with the appropriate restriction enzyme and re-fractionate it by chromatography on a fresh column of Sepharose CL-4B.

- *The cDNA inserts contain more sites for* Not*I,* Sal*I, or* Eco*RI than expected.* In mammalian DNA, *Not*I sites occur, on average, once in 1000 kb; *Sal*I sites are separated by an average of 100 kb, and *Eco*RI sites, by an average of 4 kb. If these sites occur more frequently than expected (e.g., if every cDNA carries an internal *Not*I site), it is likely that tandem arrays of cDNA molecules have been cloned into the vector. This problem can be avoided by decreasing the ratio of inserts to arms in the ligation mixture (see page 8.74).

- *The frequency of* Eco*RI sites within the cDNA inserts is less than 1 site per 4 kb.* This problem arises when methylation of *Eco*RI sites within the double-stranded cDNA is not complete. In this case, it is usually necessary to check the methylation reaction in a series of pilot reactions and then to repeat the methylation step using a fresh batch of cDNA.

- *No cDNA inserts can be excised from the bacteriophage λ vector by digestion with the appropriate restriction enzyme.* This problem is almost always caused by inefficiency in adding synthetic linkers to the ends of the double-stranded cDNA. Re-treat the ends of the double-stranded cDNA with bacteriophage T4 DNA polymerase, and repeat the large-scale ligation reaction with a mixture of unlabeled, phosphorylated linkers and ^{32}P-labeled linkers. Analyze an aliquot of the reaction by polyacrylamide gel electrophoresis (see Chapter 6) to check that the radiolabeled linkers have formed a series of ligation products that exhibit a ladder-like distribution.

  Inserts that cannot be excised by digestion with the appropriate restriction enzyme can often be recovered by digesting the recombinant bacteriophage DNA with restriction enzymes that cleave the vector sequences near the site at which the cDNA is inserted. For example, *Hin*dIII and *Bgl*II cleave on either side of the single *Eco*RI site used for cloning in λgt10, generating a DNA fragment approximately 1 kb in length. The size of cDNA inserts in putative λgt10 recombinants can be estimated from changes in the mobility of this fragment and the appearance of novel DNA fragments.

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9

Analysis and Cloning of Eukaryotic Genomic DNA

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If current estimates are correct, we shall move in the short span of 50 years from the discovery of the structure of DNA to the elucidation of the complete sequence of the human genome. Although this rapid rate of progress has been catalyzed by many theoretical and technical advances, none has been so informative as the ability to construct libraries of eukaryotic genomic DNA. Originally devised as banks from which individual bacterial genes could be isolated and analyzed (Clarke and Carbon 1976), genomic DNA libraries are now increasingly viewed as matrices that can be assembled into maps of the genome from which they were originally derived.

The feasibility of using libraries in this way was established in the late 1970s, when Maniatis et al. (1978) devised a strategy to generate and clone large numbers of random fragments of mammalian genomic DNA. Before then, genomic libraries consisted of fragments of DNA obtained by complete digestion of genomic DNA with a restriction enzyme such as *Eco*RI. The earliest libraries of mammalian genomic DNA contained only subsets of this large population of fragments because it was necessary to enrich for the sequences of interest due to the comparative inefficiency of packaging of bacteriophage λ DNA. For example, Tilghman et al. (1977) constructed a library from fragments of mouse DNA that had been enriched approximately tenfold for β-globin sequences by preparative gel electrophoresis and reversed-phase chromatography (Hardies and Wells 1976). The improvement of in vitro packaging systems (Hohn and Murray 1977; Sternberg et al. 1977) and the development of in situ hybridization techniques (Benton and Davis 1977) soon allowed much larger libraries to be constructed from unfractionated populations of restriction fragments (Maniatis et al. 1978; Smithies et al. 1978), thus eliminating the need to enrich the starting DNA for the sequences of interest. The inherent limitations of genomic DNA libraries constructed from complete digests of genomic DNA with a restriction enzyme such as *Eco*RI then quickly became apparent. These limitations include the following:

- Because of their large size, libraries of complete digests are laborious both to create and to screen. The average distance between *Eco*RI sites in the mammalian genome is approximately 4 kb (Botchan et al. 1974), and complete digests therefore contain approximately 10^6 different fragments of DNA. Consequently, a library constructed from these fragments needs to contain almost 10^7 independent recombinants to have a reasonable chance of including a desired sequence.
- If the sequence of interest contains one or more recognition sites for the particular restriction enzyme, it will be cloned in two or more nonoverlapping recombinants. In libraries of complete restriction digests, there is no way to "walk" from one recombinant clone to another that contains the neighboring restriction fragment in the original genomic DNA.
- Because of the quasi-random distribution of restriction sites in mammalian DNA, the sequence of interest may by chance be located on a fragment of genomic DNA that is too large or too small for the vector to accept.

The solution to these problems was to clone large pieces of DNA generated

either by mechanical shearing (Wensink et al. 1974; Clarke and Carbon 1976) or by partial digestion with restriction enzymes that cleave the target DNA frequently (Maniatis et al. 1978). Unfortunately, the efficiency of cloning sheared DNA was not sufficient to obtain representative libraries. Thus, virtually all of the libraries used to isolate single-copy genes were produced by partial digestion of genomic DNA with restriction enzymes. These DNA fragments were fractionated by density gradient centrifugation or preparative gel electrophoresis to select those whose size was suitable for insertion into the vector (Maniatis et al. 1978). This method, which is used in an essentially unchanged form today, helps to ensure that there is no systematic exclusion of sequences from the cloned library merely because of an unfortunate distribution of restriction sites. The following advantages are gained by constructing libraries of randomly cleaved DNA:

- *The ability to "walk" along the eukaryotic chromosome in a way that is impossible with a library of nonoverlapping restriction fragments.* Random cleavage of genomic DNA generates collections of overlapping fragments from each chromosome. Thus, a library of these fragments should consist of 46 independent collections of overlapping clones. If the library contains no gaps, the clones forming an individual series will correspond to the entire DNA sequence of an individual chromosome. To walk along a chromosome, a segment of nonrepetitive DNA derived from one end of a given recombinant is used as a probe to identify overlapping clones that contain the adjacent sequence. This process can be repeated until clones have been identified that cover the entire region of interest.
- *The ability to isolate segments of DNA without knowledge of the location of restriction sites in and around the target sequences.*
- *A reduction in the size of the library.* Because the DNA fragments selected for cloning are large, the number of recombinants required to generate a mammalian genomic DNA library is significantly reduced.

Note: The exact probability of having any given DNA sequence in the library can be calculated from the equation

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where P is the desired probability, f is the fractional proportion of the genome in a single recombinant, and N is the necessary number of recombinants (Clarke and Carbon 1976). For example, to achieve a 99% probability ($P = 0.99$) of having a given DNA sequence represented in a library of 17-kb fragments of a mammalian genome (3×10^9 bp)

$$N = \frac{\ln(1-0.99)}{\ln\left(1-\left[\frac{1.7\times10^4}{3\times10^9}\right]\right)} = 8.1\times10^5$$

Vectors Used to Construct Eukaryotic Genomic DNA Libraries

Bacteriophage λ and Cosmid Vectors

Two kinds of vectors are commonly used to construct libraries of genomic DNA: bacteriophage λ vectors and cosmids. In both cases, large segments of eukaryotic DNA, generated by quasi-random fragmentation, are ligated with vector DNA to form concatemers that can be packaged into bacteriophage λ particles. Libraries constructed in bacteriophage λ vectors are stored and propagated in the form of such recombinant bacteriophage particles. In cosmid cloning, however, these particles serve merely as vehicles to introduce the recombinant genomes into bacteria, where they are propagated as large plasmids. Because cosmids can accept approximately 45 kb of foreign DNA—almost twice as much as bacteriophage λ vectors—it is necessary to generate only 350,000 recombinant cosmids to achieve a 99% probability that a particular single-copy sequence will be represented in a library of mammalian genomic DNA. However, genomic DNA libraries are considerably more difficult to construct and maintain in cosmids than in bacteriophage λ vectors (see Chapter 3). Cosmids are therefore used only when the target gene is too large to be contained within a single bacteriophage λ recombinant or when a series of overlapping clones are to be isolated that span an extensive region of chromosomal DNA. For routine isolation of segments of eukaryotic genes and in circumstances where a library might be expected to be used many times, we recommend bacteriophage λ vectors. Further discussion of the relative merits of bacteriophage λ and cosmid vectors for the construction of libraries of genomic DNAs can be found in Chapters 2 and 3.

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The Yeast Artificial Chromosome System

Although vectors based on bacteriophage λ have been extremely powerful tools for the isolation of both cDNA and genomic versions of many eukaryotic genes, they can only accommodate inserts that fall within a defined size range. Currently available bacteriophage λ vectors can carry inserts up to 24 kb in size, and cosmid vectors can accommodate inserts approximately 35–45 kb in length. In the past few years, it has become obvious that many genes are too large to be cloned as a single fragment in these vectors. For example, the gene for human factor VIII is 180 kb in length (Gitschier et al. 1984) and the dystrophin gene is at least 1800 kb in length (Robertson 1987). Furthermore, efforts to map and clone large segments of eukaryotic chromosomes have been hindered by the small size of the individual steps that can be achieved during chromosome walking.

The need to clone large segments of DNA has been at least partially addressed by the development of yeast artificial chromosomes (YACs), which were first described by Olson and his colleagues (Burke et al. 1987; see also Cooke 1987). The development of this cloning system was made possible by several other events: (1) the identification and characterization of *cis*-acting elements required for chromosomal stability in the yeast *Saccharomyces cerevisiae* (see, e.g., Szostak and Blackburn 1982), (2) the development of a system to transform yeast with high efficiency (Burgers and Percival 1987), and (3) the ability to resolve very large DNA fragments (in the size range of 50 kb up to 10,000 kb) by pulsed-field gel electrophoresis (see Chapter 6). The principle of the YAC system is similar to that used in conventional cloning of genomic DNA. Large fragments of DNA are ligated into two "arms" of a YAC vector, and the ligation mixture is then introduced into the yeast by transformation. Each of the arms of the YAC vector carries a selectable marker as well as appropriately oriented DNA sequences that function as telomeres in yeast. In addition, one of the two arms carries two small DNA fragments that function as a centromere and as an origin of replication (also called an ARS element—*a*utonomously *r*eplicating *s*equences). Yeast transformants that have taken up and stably maintained an artificial chromosome are identified as colonies on agar plates containing the components necessary for selection of one or both YAC arms. YAC vectors are designed to allow rapid identification of transformants that carry inserts of genomic DNA. Insertion of DNA into the cloning site interrupts a suppressor tRNA gene and results in the formation of red rather than white colonies by yeast strains that carry an amber *ade*2 gene.

To clone in YAC vectors, genomic DNA from the test organism is carefully prepared under conditions that result in relatively little shearing, such that its average size is several million base pairs. The DNA is then partially digested with a restriction enzyme, such as *Eco*RI, to generate fragments whose average size is 200–500 kb. The fragments are then ligated to the arms of the YAC vector, which has been appropriately prepared to prevent self-ligation. As an alternative to partial digestion with *Eco*RI, YAC vectors may be used that will accept genomic DNA that has been digested to completion with rarely cutting restriction enzymes such as *Not*I or *Mlu*I.

The transformation system to introduce YACs into yeast spheroplasts results in approximately 500 transformants per microgram of ligated genomic

DNA. Although this is orders of magnitude lower in efficiency than bacterial transformation, it is nevertheless sufficient to allow construction of complete mammalian genomic DNA libraries in YACs. Overlapping libraries, generated by partial digestion of mammalian DNA, can be used for chromosome walking in the same way that genomic DNA libraries constructed in bacteriophage λ or cosmid vectors can be used. Libraries of DNA that has been digested to completion with *Not*I or *Mlu*I obviously do not contain overlapping clones, but they may allow the isolation of a specific segment of DNA of large size.

Because the YAC system is relatively new, there have been few published reports of either successes or possible problems. For example, the optimal and maximum sizes of YACs are not yet known, and no information is available on the extent to which repetitive mammalian DNA sequences may rearrange during extended propagation in yeast. However, even with these limitations, it is already clear that the YAC cloning system will provide an important new tool for isolating and characterizing large regions of mammalian genomes.

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BACTERIOPHAGE λ VECTORS RECOMMENDED FOR CONSTRUCTION OF LIBRARIES OF EUKARYOTIC GENOMIC DNA

The first generation of bacteriophage λ vectors, such as Charon 4A (see Chapter 2, page 2.19) and λgt-WES (see Maniatis et al. 1982 for map), which were developed in the late 1970s, could accept segments of foreign DNA approximately 15–20 kb in length. The genomic DNA libraries constructed in these vectors were the source of most of the cloned segments of mammalian genomic DNA isolated before 1983, and some of these libraries are used occasionally even today. However, de novo construction of genomic DNA libraries is now carried out using more modern vectors (e.g., the EMBL series, λ2001, λDASH, and Charon 38–40 [see Chapter 2, pages 2.30–2.37]) that can propagate up to approximately 24 kb of foreign DNA. This increased capacity allows a modest reduction in the number of recombinant clones that need be constructed and screened to isolate a particular segment of the eukaryotic genome. More significant, however, are the cumulative savings of time and effort during the several rounds of screening that are required to isolate a set of overlapping clones spanning a particular region of genomic DNA. Factors to consider in choosing among the available vectors with high capacity are:

- *The ease with which inserts of foreign DNA can be recovered from the recombinant bacteriophages.* Most modern substitution vectors carry two identical polycloning sites located in opposite orientations at each end of the stuffer fragment. Usually, the arms of these vectors are prepared with a restriction enzyme that cleaves the polycloning sites at positions proximal to the stuffer fragment. Segments of foreign DNA can subsequently be retrieved by cleaving the DNA of recombinant bacteriophages with another restriction enzyme that recognizes one of the flanking sites in the polycloning site. This can be a great boon when subcloning or constructing restriction maps of segments of foreign DNA. The number and order of restriction sites in polycloning sites varies from vector to vector. As more sophisticated vectors have been developed, the number and variety of useful cloning sites has increased dramatically, thereby broadening the choice of enymes that may be used to prepare the genomic DNA for cloning and to recover fragments from specific recombinants.
- *The ease with which arms can be prepared.* A few bacteriophage λ vectors have been designed to allow recombinants to be selected by growth in certain bacterial hosts. For example, recombinants constructed in vectors of the EMBL series can be selected by virtue of their Spi^- phenotype (see Chapter 2, page 2.12). Consequently, when using vectors of this type, there is no need to purify the arms of the bacteriophage λ DNA before constructing genomic DNA libraries. However, when constructing libraries in vectors that do not allow genetic selection of recombinants, it is necessary to purify the arms in order to minimize the number of nonrecombinant bacteriophages. Several recently developed vectors carry features that simplify the task of preparing arms (e.g., precipitation with polyethylene glycol for Charon 39 and 40 [Dunn and Blattner 1987]). Although these methods are elegant, they should not dictate the choice of vector. The speed of preparation is far less important than the quality of the arms. In our

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experience, removal of the stuffer fragment by the classical method of density gradient centrifugation (Chapter 2, pages 2.85–2.89) yields arms that can be efficiently ligated to foreign DNA and does not yield a high proportion of nonrecombinant bacteriophages. The arms of many vectors are available from commercial sources, and it may be cost-effective for those planning to make only one genomic DNA library to purchase, rather than make, arms. However, because the labor involved in purifying arms is relatively small, it does not make sense to buy arms if they are to be used regularly.

- *The characterization of the vector.* The complete DNA sequences of the arms of most vectors in current use have been determined or can be deduced from the established sequence of wild-type bacteriophage λ DNA (Sanger et al. 1982; Daniels et al. 1983). Knowledge of the sequence can be a great help in devising strategies to characterize and map cloned segments of foreign DNA. In some cases, the sequence of the stuffer fragment may not be available, but this is usually not relevant.

- *The presence of amber mutations in the arms.* Amber mutations are carried in essential genes in many currently used vectors (or their derivatives). This allows genomic DNA libraries to be screened for sequences of interest by the elegant recombination system developed by Seed (1983) and improved by Lutz et al. (1987). Although this method is used only rarely, it is extremely powerful. It is therefore advisable to keep open the option of screening by recombination by using, if possible, a vector that carries two or more amber mutations.

- *The presence of an active* gam *gene in the recombinant.* As described in Chapter 2, pages 2.12–2.13, the product of the bacteriophage λ *gam* gene inhibits the host-encoded *recBC* nuclease (Friedman and Hays 1986). In *recA*$^-$ bacteria, therefore, the rolling-circle DNA concatemers of *gam*$^+$ recombinants are not degraded and production of progeny bacteriophage particles is not suppressed. The ability to propagate genomic DNA libraries in *recA*$^-$ hosts reduces the likelihood of rearrangement or loss of foreign DNA sequences by recombination. Particularly vulnerable to rearrangement are genomic DNA fragments that include head-to-head inverted repetitions. Recombinant bacteriophages carrying such segments cannot be propagated on *rec*$^+$ hosts (Leach and Stahl 1983; Nader et al. 1985; Wyman et al. 1985; Leach and Lindsey 1986; Wertman et al. 1986). The more recently developed vectors of the Charon series (Charon 38, 39, and 40) carry the *gam* gene in the right arm (Dunn and Blattner 1987) and therefore may have some advantages for cloning inverted repeat sequences because they give rise to *gam*$^+$ recombinants that can be propagated on *recA*$^-$ hosts. Because the product of the *gam* gene inhibits the *Escherichia coli recBC* nuclease, cells infected with *gam*$^+$ recombinant bacteriophages are phenotypically RecBC$^-$.

- *The presence of a* chi *sequence in the arms of the vector. chi* is an 8-bp sequence that stimulates *E. coli recBC*-dependent recombination and thereby stimulates the growth of *red*$^-$ *gam*$^-$ recombinants. Within a genomic DNA library constructed in a *red*$^-$ *gam*$^-$ vector lacking *chi,* there are marked differences in plaque size depending on whether or not a cloned

genomic DNA segment happens to contain a *chi* sequence. A solution to this problem is to include a *chi* sequence in one of the arms of the vector. The presence of this sequence decreases by a factor of 20 the number of recombinant bacteriophages that cannot be propagated on *rec*$^+$ hosts (Wyman et al. 1986). Among the vectors that carry a *chi* sequence are EMBL3 and 4, λ2001, λFIX and λDASH. (For further discussion of *chi* sequences, see Chapter 2, page 2.13.)

The basic steps used in the construction of libraries of random fragments of eukaryotic DNA in bacteriophage λ vectors are shown in Figure 9.1 and are summarized as follows:

1. The DNA of a substitution vector of bacteriophage λ that will accept 20–24-kb segments of foreign DNA is digested with a restriction enzyme. The left and right arms, which carry all of the genetic information essential for lytic growth of the virus, are separated from the internal stuffer fragment by any of several methods (fractionation by size, digestion with restriction enzymes, differential precipitation, etc.).
2. High-molecular-weight eukaryotic DNA is partially digested with a restriction enzyme, or a combination of enzymes, that recognizes and cleaves at a specific sequence of four nucleotides (see pages 9.24–9.28). Because the number of potential target sites in the eukaryotic genome for enzymes of this type is very large, partial digests will contain overlapping fragments that form an overlapping series colinear with the original genome.
3. Fragments of genomic DNA suitable for cloning in bacteriophage λ vectors can be prepared from the partial digest in one of two ways:
   - Fragments of the appropriate length (20–24 kb) can be selected from the population by gel electrophoresis or density gradient centrifugation (see page 9.27). This step significantly reduces the possibility of cloning more than one genomic DNA fragment in each bacteriophage λ vector.
   - The recessed termini of the digested DNA can be partially filled using the Klenow fragment of *E. coli* DNA polymerase I (see page 9.29 and Figure 9.1B). For example, the first two nucleotides of the termini created by partial digestion of genomic DNA with *Sau*3AI can be filled with dGTP and dATP. This creates short protruding termini that are no longer self-complementary (Zabarovsky and Allikmets 1986). It is therefore extremely unlikely that partially digested and filled fragments will become ligated into structures containing segments of DNA that were noncontiguous in the original genome. This greatly reduces the possibility of creating a library containing a high proportion of scrambled genomic clones.
4. The arms of the bacteriophage λ vector and the fragmented eukaryotic DNA are ligated together to form long concatemeric molecules that are subsequently packaged into bacteriophage λ heads using in vitro packaging systems. These arms may be prepared in several ways that maximize the efficiency of cloning:
   - If the vector carries *red* and *gam* genes in its stuffer fragment (e.g.,

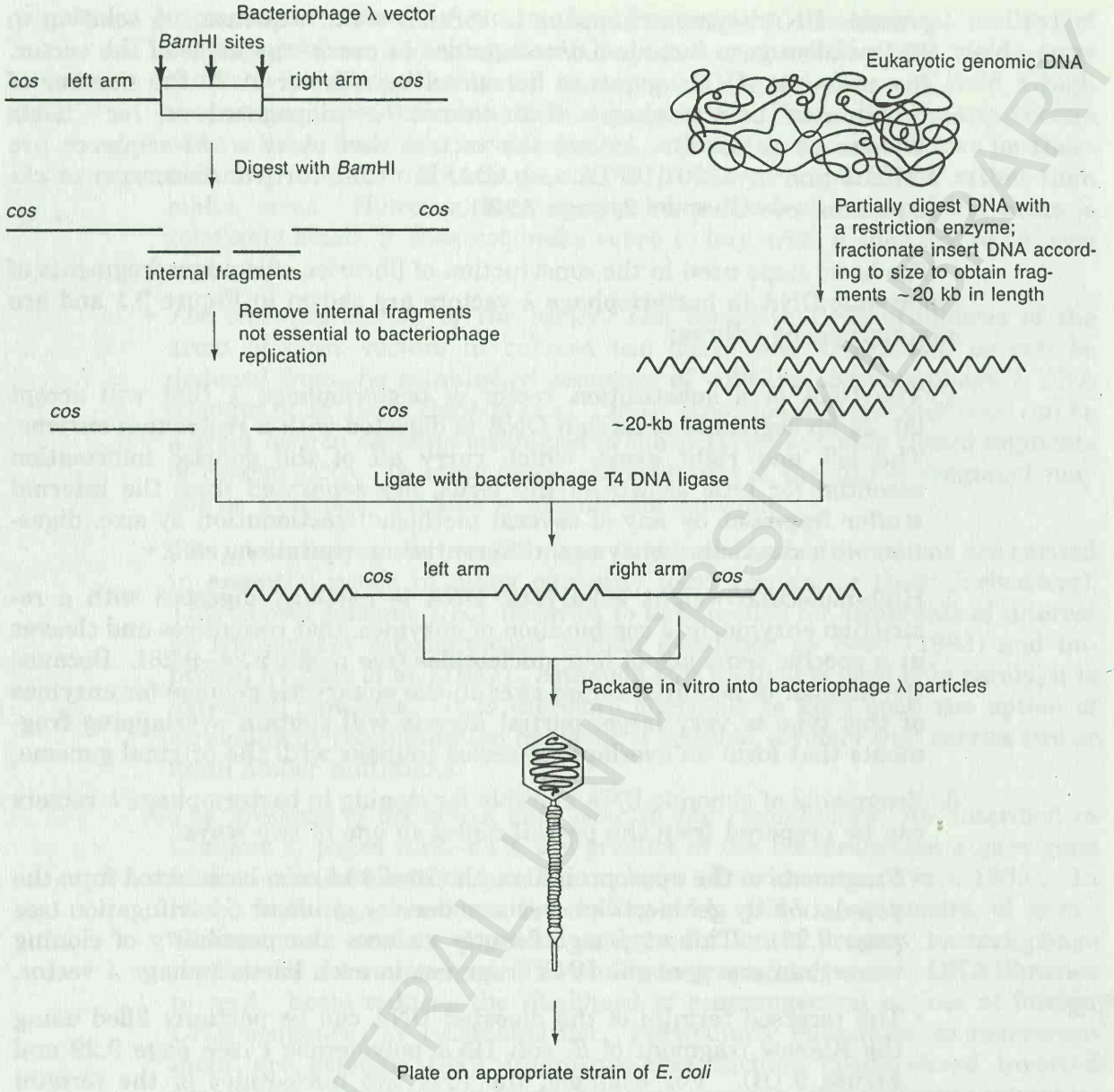

FIGURE 9.1A

A strategy used to construct libraries of random fragments of eukaryotic DNA. (*Left*) Preparation of the vector DNA fragments; (*right*) preparation of eukaryotic DNA fragments. A concatemeric recombinant DNA molecule is produced by the action of bacteriophage T4 DNA ligase. This concatemer is the substrate for the in vitro packaging reaction during which a different recombinant DNA molecule is inserted into each bacteriophage λ particle. Following amplification by growth in *E. coli,* a lysate is obtained, consisting of a library of recombinant clones that, in aggregate, contain most of the sequences present in the eukaryotic genome.

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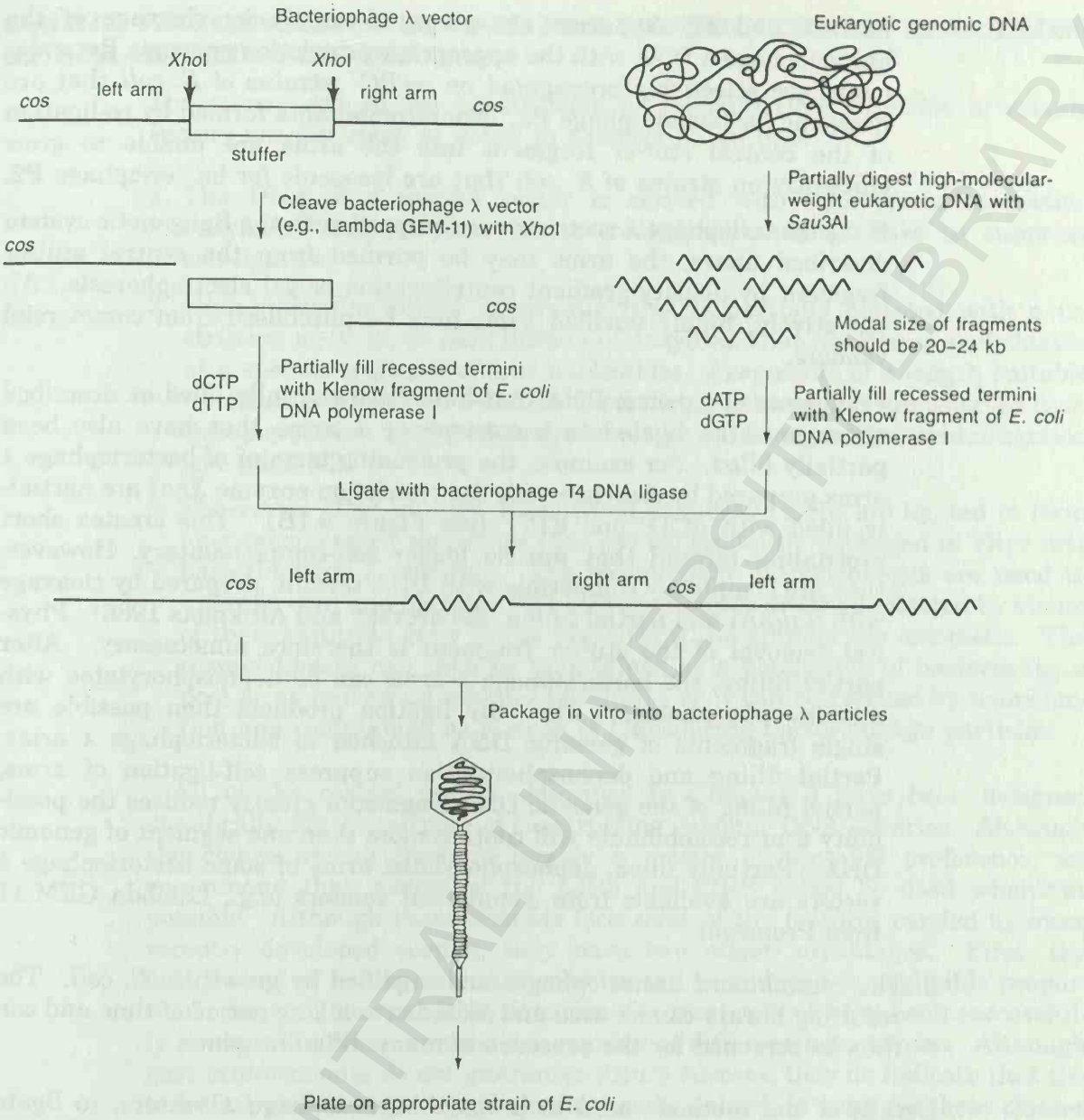

FIGURE 9.1B
Construction of libraries of unfractionated genomic DNA.

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EMBL3 and 4), the arms are prepared simply by cleavage of the bacteriophage λ DNA with the appropriate restriction enzyme. Recombinants are selectively propagated on *recBC*$^+$ strains of *E. coli* that are lysogenic for bacteriophage P2. Nonrecombinants formed by re-ligation of the central stuffer fragment into the arms are unable to grow efficiently on strains of *E. coli* that are lysogenic for bacteriophage P2.

- If the bacteriophage λ vector is not equipped with the Spi genetic system described above, the arms may be purified from the central stuffer fragment by density gradient centrifugation or gel electrophoresis. Alternatively, highly purified arms may be purchased from commercial vendors.

- Fragments of genomic DNA that have been partially filled as described above must be ligated to bacteriophage λ arms that have also been partially filled. For example, the protruding termini of bacteriophage λ arms prepared by cleavage with the restriction enzyme *Xho*I are partially filled with dCTP and dTTP (see Figure 9.1B). This creates short protruding termini that are no longer self-complementary. However, they are completely compatible with DNA termini prepared by cleavage with *Sau*3AI and partial filling (Zabarovsky and Allikmets 1986). Physical removal of the stuffer fragment is therefore unnecessary. After partial filling, the bacteriophage λ arms can be dephosphorylated with alkaline phosphatase. The only ligation products then possible are single fragments of genomic DNA attached to bacteriophage λ arms. Partial filling and dephosphorylation suppress self-ligation of arms; partial filling of the genomic DNA fragments greatly reduces the possibility that recombinants will contain more than one segment of genomic DNA. Partially filled, dephosphorylated arms of some bacteriophage λ vectors are available from commercial vendors (e.g., Lambda GEM-11 from Promega).

5. The recombinant bacteriophages are amplified by growth in *E. coli*. The resulting library can be used and stored over a long period of time and can thus be screened for the presence of many different genes.

Details of the methods used to prepare bacteriophage λ vectors, to ligate them to appropriately sized fragments of genomic DNA, to package the resulting molecules into bacteriophage particles, and to establish and maintain genomic DNA libraries are given in Chapter 2.

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COSMID VECTORS RECOMMENDED FOR CONSTRUCTION OF LIBRARIES OF EUKARYOTIC GENOMIC DNA

The steps used in the construction of genomic DNA libraries in cosmid vectors are discussed in detail in Chapter 3. In brief:

1. The DNA of a cosmid vector is cleaved with appropriate restriction enzyme(s) and, in some cases, treated with phosphatase to suppress self-ligation.
2. High-molecular-weight eukaryotic DNA is partially digested with a restriction enzyme, or combination of enzymes, that recognizes and cleaves at a specific sequence of four nucleotides. Fragments of a length suitable for cloning in cosmids (~45 kb depending on the vector) are selected from this population by gel electrophoresis or density gradient centrifugation (see page 9.27).
3. The vector DNA and the fragmented eukaryotic DNA are ligated to form long concatemeric molecules that are subsequently packaged in vitro into bacteriophage λ particles. The recombinant bacteriophages are used to infect *E. coli*, and bacteria that contain cosmids are then selected by virtue of their ability to grow in the presence of the appropriate antibiotic. The cosmid library can then be maintained as a population of bacteria (as a pool or as individual colonies on filters), or it can be rescued by transduction and maintained as a stock of transducing bacteriophage particles.

All of the cosmid vectors described in Chapter 3 have been designed specifically for the purpose of constructing genomic DNA libraries. Although the choice among them is largely a matter of personal preference, we recommend that vectors of the pJB8 and c2RB series be used whenever possible. Although these cosmids lack some of the features carried by more recently developed vectors, they have two major advantages. First, the background of nonrecombinant colonies can be reduced to negligible proportions. Second, genomic DNA libraries of several species have been successfully produced in these vectors in a number of different laboratories. Although past achievements do not guarantee future success, they do indicate that the technical problems of cosmid cloning can be solved, at least for these classes of vectors. Other cosmids should be used only when specifically required by the experiment, for example, when the vector must carry a selectable marker for mammalian cells.

In the remainder of this chapter, we describe ways to isolate and fragment eukaryotic DNA preparatory to cloning. The resulting fragments can be used to construct genomic DNA libraries in either bacteriophage λ or cosmid vectors, according to the experimenter's choice. Details of the methods used to prepare bacteriophage λ and cosmid vectors, to ligate them to appropriately sized fragments of genomic DNA, to package the resulting molecules into bacteriophage particles, and to establish and maintain genomic DNA libraries are given in Chapters 2 and 3.

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Isolation of High-molecular-weight DNA from Mammalian Cells

Mammalian DNA is usually isolated by digestion of cells with proteinase K in the presence of EDTA and a detergent such as SDS, followed by extractions with phenol. Low-molecular-weight contaminants are removed by dialysis. This method yields DNA whose size (100–150 kb) is adequate for Southern analysis and for construction of genomic DNA libraries in bacteriophage λ. The successful construction of libraries in cosmids, however, requires larger DNA. Fragments of DNA with one sheared end and one restriction-enzyme-generated end compete for the cosmid DNA in the ligation reaction and effectively prevent the formation of concatemers that can be packaged into bacteriophage λ particles. To avoid this problem, the starting DNA needs to be severalfold longer than the partial digestion products used to construct the library. Figure 9.2 shows the relationship between the fraction of partial digestion products that carry two termini suitable for cloning and the initial size of the genomic DNA. The yield of useful fragments is relatively poor unless the initial length of the DNA is at least fourfold greater than that of the fragments used for cloning. For construction of genomic DNA libraries in cosmids, the length of the starting DNA should therefore exceed 200 kb. It is difficult to isolate DNA of this size by methods that involve extraction with organic solvents and mechanical shearing, as this is where most loss of DNA occurs. Below, we describe three procedures to isolate DNA from mammalian cells and tissues. The first (Blin and Stafford 1976), a modification of the original procedure (Gross-Bellard et al. 1973), uses proteinase K, SDS, and phenol and yields DNA whose size (100–150 kb) is adequate for production of genomic DNA libraries in bacteriophage λ vectors. The second method involves digestion with proteinase K, dissociation of DNA-protein complexes with high concentrations of formamide, and removal of residual proteinase K by dialysis through collodion bags (Kupiec et al. 1987). This method takes longer than the first but avoids the labor-intensive step of extraction with organic solvents. Because the DNA is neither shaken nor pipetted nor precipitated with ethanol, its molecular weight is very high. However, the concentration of DNA in the final preparation is often quite low (~ 10 μg/ml). The third method, which uses guanidine HCl to disrupt cells, yields DNA whose size is rather small (~ 80 kb). However, the method is very rapid and can be used to extract DNA simultaneously from many different cells or tissues (Bowtell 1987). Although DNA prepared by this method should not be used to construct genomic DNA libraries, it gives excellent results in Southern hybridizations.

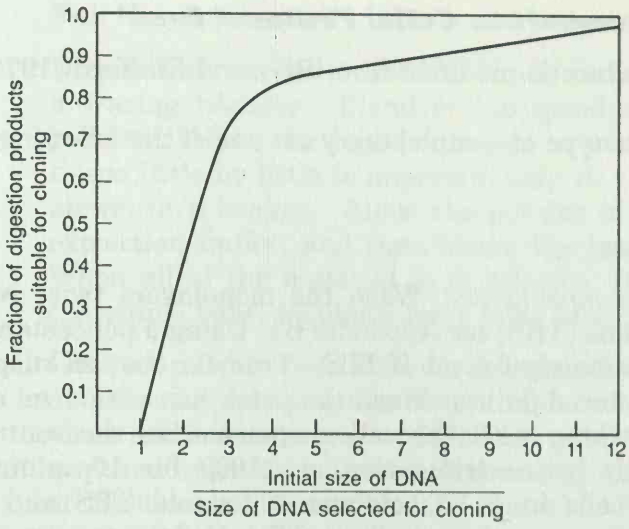

FIGURE 9.2
The relationship between the fraction of partial digestion products that carry two termini suitable for cloning and the initial size of the genomic DNA.

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Isolation of DNA from Mammalian Cells: Protocol I

The following procedure is modified from Blin and Stafford (1976).

1. Depending on the type of sample, carry out one of the following procedures as step 1.

For cell samples

Cells growing in monolayers: Wash the monolayers twice with ice-cold Tris-buffered saline (TBS; see Appendix B). Using a policeman, scrape the cells into approximately 0.5 ml of TBS. Transfer the cell suspension to a centrifuge tube stored on ice. Wash the petri dish with 1 ml of TBS, and combine the washing with the cell suspension in the centrifuge tube. Recover the cells by centrifugation at 1500*g* for 10 minutes at 4°C. Resuspend the cells in 5–10 volumes of ice-cold TBS and repeat the centrifugation. Resuspend the cells in TE (pH 8.0) at a concentration of 5×10^7 cells/ml. Transfer the solution to an Erlenmeyer flask. (For 1 ml of cell suspension, use a 50-ml flask; for 2 ml, use a 100-ml flask; etc.) Add 10 ml of extraction buffer for each milliliter of cell suspension. Incubate the solution for 1 hour at 37°C, and then proceed to step 2.

Extraction buffer

10 mM Tris · Cl (pH 8.0)
0.1 M EDTA (pH 8.0)
20 μg/ml pancreatic RNAase
0.5% SDS

It is important that the cells be well-dispersed over the inner surface of the Erlenmeyer flask when the extraction buffer is added. This minimizes the formation of intractable lumps of DNA.

Blin and Stafford (1976) recommend the use of 0.5 M EDTA (pH 8.0) in the extraction buffer. However, the density of this solution almost equals that of phenol, which makes separation of the phases in step 4 difficult. In our hands, 0.1 M EDTA (pH 8.0) is equally effective in maintaining high-molecular-weight DNA and permits easier separation of the phenolic phase.

Pancreatic RNAase is not highly active in the presence of 0.5% SDS, but when added at high concentrations it works well enough to degrade most of the cellular RNA. Adding RNAase at this stage eliminates the need to treat semipurified DNA with the enzyme subsequently. To eliminate contaminating DNA, use RNAase that has been treated as described in Appendix B.

Cells growing in suspension: Recover the cells by centrifugation at 1500*g* for 10 minutes at 4°C. Resuspend the cells in a volume of ice-cold TBS equal to the volume of the original culture. Recover the cells by re-centrifugation, and repeat the washing procedure. Resuspend the cells in TE (pH 8.0) at a concentration of 5×10^7 cells/ml. Transfer the suspension to an Erlenmeyer flask of the appropriate size (see above). Add extraction buffer and incubate as described above.

For tissue samples

Drop freshly excised tissue into liquid nitrogen in the stainless-steel cup of a Waring Blendor. Blend at top speed until the tissue is ground to a powder. Allow the liquid nitrogen to evaporate, and add the powdered tissue little by little to approximately 10 volumes of extraction buffer (see above) in a beaker. Allow the powder to spread over the surface of the extraction buffer, and then shake the beaker to submerge the material. When all of the material is in solution, transfer the solution to a 50-ml centrifuge tube, incubate for 1 hour at 37°C, and then proceed to step 2.

Caution: It has been well-documented that human blood, blood products, and tissues may contain occult infectious materials such as hepatitis B virus and human immunodeficiency virus that may result in laboratory-acquired infections. Investigators using any human blood, blood products, or tissues should consider the materials biohazardous and handle them accordingly until proven otherwise. Appropriate measures include: wearing disposable gloves, using mechanical pipetting devices, working in a biological safety cabinet whenever there is the possibility of aerosol generation, and disinfection of all waste materials prior to disposal. Consult your institutional safety officer for specific handling and disposal procedures.

For blood samples

Collect approximately 20 ml of fresh blood in tubes containing 3.5 ml of acid citrate dextrose solution B (ACD). This anticoagulant is superior to EDTA in preserving high-molecular-weight DNA during storage of blood (Gustafson et al. 1987). The blood may be stored at 0°C for several days or at −70°C indefinitely before the DNA is prepared. To prepare ACD, mix:

citric acid	0.48 g
sodium citrate	1.32 g
glucose	1.47 g
H_2O to 100 ml	

For use as an anticoagulant, add 1 ml of ACD to 6 ml of fresh blood.

Fresh blood (20 ml): Centrifuge at 1300*g* for 15 minutes and discard the supernatant plasma. Using a pasteur pipette, carefully transfer the buffy coat to a fresh tube and recentrifuge. The buffy coat is a broad band of white blood cells of heterogeneous density. Resuspend the buffy coat obtained from this second centrifugation in 15 ml of extraction buffer (see above). Incubate the solution for 1 hour at 37°C, and proceed to step 2.

Frozen blood (20 ml): Thaw in a water bath at room temperature, transfer to a centrifuge tube, and dilute with 1 volume of phosphate-buffered saline (PBS; see Appendix B). Then centrifuge the blood at 3500*g* for 15 minutes at room temperature and discard the supernatant, which contains lysed red cells. Resuspend the pellet in 15 ml of extraction buffer (see above). Incubate the solution for 1 hour at 37°C, and then proceed to step 2.

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2. Add proteinase K to a final concentration of 100 μg/ml. Using a glass rod, gently mix the enzyme into the viscous solution.

   Proteinase K is stored as a stock solution at a concentration of 20 mg/ml in H_2O (see Appendix B).

3. Place the suspension of lysed cells in a water bath for 3 hours at 50°C. Swirl the viscous solution periodically.

4. Cool the solution to room temperature, and, if necessary, pour the solution into a centrifuge tube. Add an equal volume of phenol equilibrated with 0.5 M Tris · Cl (pH 8.0) (see Appendix B) and *gently* mix the two phases by *slowly* turning the tube end over end for 10 minutes. If the two phases have not formed an emulsion at this stage, place the tube on a roller apparatus for 1 hour. Separate the two phases by centrifugation at 5000*g* for 15 minutes at room temperature.

   It is essential that the pH of the phenol be approximately 8.0 to prevent DNA from becoming trapped at the interface between the organic and aqueous phases.

5. With a wide-bore pipette (0.3-cm-diameter orifice), transfer the viscous aqueous phase to a clean centrifuge tube and repeat the extraction with phenol twice.

   When transferring the aqueous phase, it is essential to draw the DNA into the pipette very slowly to avoid disturbing the material at the interface. If the DNA solution is so viscous that it cannot easily be drawn into a wide-bore pipette, use a long pipette attached to a water-suction vacuum pump to remove the organic phase. *Make sure that the phenol is collected into traps and does not enter the water line.*

   With the vacuum line closed, slowly lower the pipette to the bottom of the organic phase. Wait until the viscous thread of aqueous material detaches from the pipette, and then carefully open the vacuum line and gently withdraw all of the organic phase. Close the vacuum line and quickly withdraw the pipette through the aqueous phase. Immediately open the vacuum line to transfer the residual phenol into the trap. Centrifuge the DNA solution at 5000*g* for 20 minutes at room temperature. Protein and clots of DNA sediment to the bottom of the tube. Pour the DNA solution into a 50-ml centrifuge tube, leaving behind the protein and clots of DNA.

6. *To isolate very-high-molecular-weight DNA (~200 kb):* After the third extraction with phenol, dialyze the pooled aqueous phases at 4°C four times against 4 liters of a solution of 50 mM Tris · Cl (pH 8.0), 10 mM EDTA (pH 8.0) until the OD_{270} of the dialysate is less than 0.05. Allow room in the dialysis bag for the volume of the sample to increase 1.5- to 2.0-fold. Continue at step 7.

   To isolate DNA whose size is 100–150 kb: After the third extraction with phenol, transfer the pooled aqueous phases to a fresh centrifuge tube and add 0.2 volume of 10 M ammonium acetate. Add 2 volumes of ethanol at room temperature and swirl the tube until the solution is thoroughly mixed. The DNA will immediately form a precipitate that can usually be removed from the ethanolic solution with a pasteur pipette whose end has been sealed and shaped into a U. Most of the contaminating oligoribonucleotides are left behind. If the DNA precipitate becomes fragmented, collect it by centrifugation at 5000*g* for 5 minutes at room temperature in

a swinging-bucket rotor. Wash the DNA precipitate twice with 70% ethanol, and collect the DNA by centrifugation as described above. Remove as much as possible of the 70% ethanol, and store the pellet in an open tube at room temperature until the last visible traces of ethanol have evaporated. *Do not allow the pellet of DNA to dry completely; otherwise, it will be very difficult to dissolve.*

Add 1 ml of TE (pH 8.0) for each $\sim 5 \times 10^6$ cells. Place the tube on a rocking platform and gently rock the solution until the DNA has completely dissolved. This usually takes 12–24 hours.

7. Measure the absorbance of the DNA at 260 nm and 280 nm. The ratio of A_{260} to A_{280} should be greater than 1.75. A lower ratio is an indication that significant amounts of protein remain in the preparation. In this case, add SDS to a concentration of 0.5% and then repeat steps 2–7.

8. Calculate the concentration of the DNA (a solution with an OD_{260} of 1 contains approximately 50 μg of DNA per milliliter), and analyze an aliquot by pulsed-field gel electrophoresis or by electrophoresis through a 0.3% agarose gel poured on a 1% agarose support (see Chapter 6, pages 6.9–6.13). The DNA should be larger than 100 kb in size and should migrate more slowly than linear dimeric molecules of intact bacteriophage λ DNA. Store the DNA at 4°C.

Notes

i. Starting with 5×10^7 cultured aneuploid mammalian cells (e.g., HeLa cells), this method should yield approximately 200 μg of high-molecular-weight DNA. The usual yield of DNA from 20 ml of normal blood is approximately 250 μg.

ii. High-molecular-weight markers can be synthesized rapidly by incubating linear bacteriophage λ DNA (20 μg/ml) for 30 minutes at 16°C in 1× bacteriophage T4 DNA ligase buffer (see Appendix B) containing 1 mM ATP and 2 Weiss units/ml bacteriophage T4 DNA ligase.

Before ligation, heat the bacteriophage λ DNA to 56°C for 5 minutes in TE (pH 7.6) to melt the cohesive termini. At the end of the ligation reaction, add EDTA to a final concentration of 0.01 M and heat the solution to 65°C for 15 minutes to inactivate the ligase.

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Isolation of DNA from Mammalian Cells: Protocol II

This procedure is a modification of that of Kupiec et al. (1987).

1. To isolate DNA from cells, tissues, or blood, carry out steps 1–3 as described on pages 9.16–9.18.

2. Cool the solution to 15°C, and then add 10 ml of denaturation buffer.

   Denaturation buffer

   80% formamide
   0.8 M NaCl
   20 mM Tris · Cl (pH 8.0)

   Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

   Mix the solution gently using a glass rod, and then store the solution for 12 hours at 15°C.

3. Pour the viscous solution into collodion bags (Sartorius SM 13200E or equivalent). Dialyze the solution three times against 4 liters of 0.1 M NaCl, 20 mM Tris · Cl (pH 8.0), 10 mM EDTA (pH 8.0) and six times against 4 liters of 10 mM NaCl, 10 mM Tris · Cl (pH 8.0), 0.5 mM EDTA (pH 8.0).

4. Measure the absorbance of the DNA at 260 nm and 280 nm. The ratio of A_{260} to A_{280} should be greater than 1.75. If it is not, repeat steps 2–4.

5. Calculate the concentration of the DNA (a solution with an OD_{260} of 1 contains approximately 50 μg of DNA per milliliter), and analyze an aliquot by pulsed-field gel electrophoresis or by electrophoresis through a 0.3% agarose gel poured on a 1% agarose support (see Chapter 6, pages 6.9–6.13). The DNA should be larger than 200 kb in size and should migrate more slowly than linear tetrameric molecules of intact bacteriophage λ DNA. Store the DNA at 4°C.

Notes

i. Starting with 5×10^7 cultured aneuploid mammalian cells (e.g., HeLa cells), this method should yield 20 ml of a solution containing approximately 5–10 μg of high-molecular-weight DNA per milliliter. Although the concentration of DNA is relatively low, the viscosity of the solution is high because of the large size of the DNA molecules.

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ii. High-molecular-weight markers can be synthesized rapidly by incubating linear bacteriophage λ DNA (20 μg/ml) for 30 minutes at 16°C in 1× bacteriophage T4 DNA ligase buffer (see Appendix B) containing 1 mM ATP and 2 Weiss units/ml bacteriophage T4 DNA ligase.

Before ligation, heat the bacteriophage λ DNA to 56°C for 5 minutes in TE (pH 7.6) to melt the cohesive termini. At the end of the ligation reaction, add EDTA to a final concentration of 0.01 M and heat the solution to 65°C for 15 minutes to inactivate the ligase.

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Isolation of DNA from Mammalian Cells: Protocol III

This method, which is adapted from Bowtell (1987), is used to prepare DNA simultaneously from many different samples of cells or tissues. The DNA is generally too small (~80 kb) for efficient construction of genomic DNA libraries, but it gives excellent results in Southern hybridizations.

1. Prepare cell suspensions (or frozen cell powders) as described in step 1 on page 9.16.

2. Transfer the cell suspensions to centrifuge tubes, and add 7.5 volumes of lysis solution consisting of 6 M guanidine HCl ($M_r = 95.6$), 0.1 M sodium acetate (pH 5.5).

   If the DNA is to be extracted from tissues, add the frozen cell powders little by little to approximately 7.5 volumes of lysis solution in beakers. Allow the powders to spread over the surface of the lysis solution, and then shake the beakers to submerge the material. When all the material is in solution, transfer the solutions to centrifuge tubes.

3. Close the tops of the tubes and incubate for 1 hour at room temperature on a rocking platform.

4. Dispense 18 ml of ethanol at room temperature into each of a series of disposable 50-ml polypropylene centrifuge tubes. Using wide-bore pipettes, carefully layer the cell suspensions under the ethanol.

5. Recover the DNA from each tube by slowly stirring the interface between the cell lysate and the ethanol with a pasteur pipette whose end has been sealed and bent into a U shape. The DNA will adhere to the pasteur pipette, forming a gelatinous mass. Continue stirring until the ethanol and the aqueous phase are thoroughly mixed.

6. Transfer each pasteur pipette, with its attached DNA, to a separate polypropylene tube containing 5 ml of ethanol at room temperature. Leave the DNA submerged in the ethanol until all of the samples have been processed.

7. Remove each pipette, with its attached DNA, and allow as much ethanol as possible to drain away. By this stage, the DNA should have shrunk into a tightly packed, dehydrated mass, and it is often possible to remove most of the free ethanol by capillary action by touching the U-shaped end of the pipette to a stack of Kimwipes. Before all of the ethanol has evaporated from the DNA, transfer the pipette into a fresh polypropylene tube containing 5 ml of ethanol at room temperature.

8. When all of the samples have been processed, remove each pipette, with its attached DNA, and remove as much ethanol as possible as described in step 7 above. *Do not allow the pellet of DNA to dry completely; otherwise, it will be very difficult to dissolve.*

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9. Transfer each pipette to a fresh polypropylene tube containing 1 ml of TE (pH 8.0). Allow the DNAs to rehydrate by storing the tubes overnight at 4°C.

10. By the next morning, the DNAs will have become highly gelatinous but will still be attached to their pipettes. Using fresh, sealed pasteur pipettes as scrapers, gently free the pellets of DNA from their pipettes. Discard the pipettes, leaving the DNAs floating in the TE. Close the tops of the tubes and incubate the DNAs at 4°C on a rocking platform until they are completely dissolved. This often takes 24–48 hours.

11. Analyze an aliquot by pulsed-field gel electrophoresis or by electrophoresis through a 0.3% agarose gel poured on a 1% agarose support (see Chapter 6, pages 6.9–6.13). The DNA should be ~80 kb in size and should migrate more slowly than monomers. Store the DNA at 4°C.

Notes

i. DNA made by this procedure is always contaminated with a small amount of RNA. It is therefore necessary to estimate the concentration of DNA in the final preparation either by fluorimetry (see Appendix E) or by gel electrophoresis and staining with ethidium bromide (see Chapter 6). If desired, the amount of contaminating RNA can be minimized by transferring the rehydrated pellet of DNA (step 10) to a fresh polypropylene tube containing 1 ml of TE (pH 8.0) before scraping it from the pasteur pipette. This is a hazardous procedure, since there is a risk that the DNA will slide off the pipette during transfer.

ii. DNA prepared from homogenized tissues by this method often has a faint brownish-red color. This does not inhibit digestion with restriction enzymes.

iii. Starting with 1.5×10^7 cultured aneuploid mammalian cells (e.g., HeLa cells), this method should yield 1 ml of a solution containing approximately 70 μg of DNA.

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Partial Digestion of High-molecular-weight Eukaryotic DNA with Restriction Enzymes

The only method by which DNA can be fragmented in truly random fashion, irrespective of its base composition and sequence, is mechanical shearing. However, DNA prepared in this way requires several additional enzymatic manipulations (repair of termini, methylation, ligation to linkers, digestion of linkers) to generate cohesive termini compatible with those of the vectors used to generate genomic DNA libraries (Maniatis et al. 1978). On the other hand, partial digestion with restriction enzymes that recognize frequently occurring tetranucleotide sequences within eukaryotic DNA yields a population of fragments that is close to random and yet can be cloned directly.

Fragments of eukaryotic DNA suitable for the construction of genomic DNA libraries are prepared as follows:

- Carry out pilot experiments to establish conditions for partial digestion of eukaryotic DNA. The aim is to maximize the yield of fragments whose size is appropriate for insertion into a bacteriophage λ or cosmid vector.
- Guided by the results of the pilot experiments, digest a large amount of eukaryotic DNA and purify fragments of the desired size by density gradient centrifugation.

Pilot Experiments

1. Dilute 30 μg of high-molecular-weight eukaryotic DNA (>200 kb; see pages 9.16–9.19) to 900 μl with 10 mM Tris · Cl (pH 8.0) and add 100 μl of the appropriate 10× restriction enzyme buffer.

   If the concentration of the high-molecular-weight DNA is low, increase the volume of the pilot reactions and concentrate the DNA after digestion by precipitation with ethanol. Each pilot reaction should contain at least 1 μg of DNA to allow the heterogeneous products of digestion to be detected by staining with ethidium bromide.

   When constructing libraries in cosmid vectors, the eukaryotic DNA should be at least 200 kb in length; for bacteriophage λ vectors, the DNA should be larger than 100 kb in length. The size of the DNA should be checked by pulsed-field gel electrophoresis or by electrophoresis through a 0.3% agarose gel poured on a 1% agarose support (see Chapter 6), using as markers oligomers of bacteriophage λ DNA. These markers can be synthesized rapidly by incubating linear bacteriophage λ DNA (20 μg/ml) for 30 minutes at 16°C in 1× bacteriophage T4 DNA ligase buffer (see Appendix B) containing 1 mM ATP and 2 Weiss units/ml bacteriophage T4 DNA ligase. Before ligation, heat the bacteriophage λ DNA to 56°C for 5 minutes in TE (pH 7.6) to melt the cohesive termini. At the end of the ligation reaction, add EDTA (pH 8.0) to a final concentration of 0.01 M and heat the solution to 65°C for 15 minutes to inactivate the ligase.

   Handle the eukaryotic DNA carefully by using either pipette tips that have been cut off with a sterile razor blade to enlarge the orifice or disposable wide-bore glass capillaries.

   Make sure that the DNA is dispersed homogeneously throughout the buffer used for digestion. The chief problem encountered during digestion of high-molecular-weight

DNA is unevenness of digestion caused by variations in the local concentration of DNA. Clumps of DNA are relatively inaccessible to restriction enzymes and can be digested only from the outside. Unless the DNA is evenly dispersed, the rate of digestion cannot be predicted or controlled. To ensure homogeneous dispersion of the DNA:

a. Allow the DNA to stand at 4°C for several hours after dilution and addition of 10 × restriction enzyme buffer.
b. *Gently* stir the DNA solution from time to time using a sealed glass capillary.
c. After addition of the restriction enzyme, *gently* stir the solution for 2–3 minutes at 4°C before warming the reaction to the appropriate temperature.
d. After digestion for 15–30 minutes, add a second aliquot of restriction enzyme and stir the reaction as described above.

2. Carry out test digestions on aliquots of the batch of *diluted* DNA that will be used to prepare fragments for cloning. The aim is to determine the amount of enzyme required to reduce the modal size of the DNA to approximately 45 kb (for construction of libraries in cosmids) or 23 kb (when bacteriophage λ is used as a vector). The amount of enzyme necessary will vary for each batch of enzyme and preparation of DNA.

a. Using a wide-bore glass capillary or a cut-off disposable plastic pipette tip, transfer 60 μl of the DNA solution to a microfuge tube (tube 1). Transfer 30 μl of the DNA solution to each of nine additional labeled microfuge tubes. Stand the tubes on ice.

b. Add 2 units of the appropriate restriction enzyme to the first tube. Use a sealed glass capillary to mix the restriction enzyme with the DNA. Do not allow the temperature of the reaction to rise above 4°C. Using a fresh pipette tip, transfer 30 μl of the reaction to the next tube in the series. Mix as before, and continue transferring the reaction to successive tubes. Do not add anything to the tenth tube (no enzyme control), but discard 30 μl from the ninth tube. Incubate the reactions for 1 hour at 37°C.

c. At the end of the digestion, heat the reactions to 70°C for 15 minutes to inactivate the restriction enzyme. After cooling the reactions to room temperature, add the appropriate amount of gel-loading buffer (see Appendix B). Mix the solutions gently, using a sealed glass capillary. Use a cut-off, disposable plastic pipette tip or a disposable wide-bore glass capillary to transfer the solutions to the wells of a 0.3% agarose gel poured on a 1% agarose support (see Chapter 6, pages 6.9–6.13). Compare the size of the digested eukaryotic DNA with that of oligomers of bacteriophage λ DNA and plasmids (see notes to step 1).

Notes

i. The gel should be run slowly (<1 V/cm) at 4°C to prevent smearing of the fragments of DNA.

ii. Occasionally, problems arise during loading of the gel because the DNA solution will not sink to the bottom of the well. This occurs when very high-molecular-weight DNA is present in the preparation. To minimize the problem, make sure that the DNA is homogeneously dispersed and

load the samples very slowly into the wells of the gel. After loading, allow the gel to stand for a few minutes so that the DNA can diffuse evenly throughout the wells.

iii. Before digestion, there should be no low-molecular-weight DNA detectable. With increasing time of digestion or increasing amounts of restriction enzyme, the modal size of DNA should decrease monotonically, i.e., there should be no residual material at the top of the gel and there should be no sign of a resistant fraction of DNA. Optimal conditions of digestion should yield a population of DNA molecules that migrates as a fairly compact band of the desired size. After staining the gel with ethidium bromide, the maximum intensity of fluorescence should be in the region of the gel containing DNA whose size is 45 kb (cosmids) or 23 kb (bacteriophage λ). If the DNA smears into the lower part of the gel, do not proceed further. It is likely that the DNA has been asynchronously digested by the restriction enzyme, with some molecules in the starting population being cleaved more frequently than others. This can occur if the restriction buffer and enzyme are not distributed homogeneously throughout the DNA solution at the start of the reaction. One solution to this problem is to dialyze the DNA to be used for the pilot and large-scale digests for several hours against a 20-fold excess of the appropriate 1 × restriction enzyme buffer.

iv. Other difficulties in obtaining satisfactory partial digestion of high-molecular-weight eukaryotic DNA are caused by inhibitors that are not removed by the rather mild procedure used to purify the DNA. In this case, extract the DNA once with phenol:chloroform and dialyze it *extensively* against changes of TE (pH 7.6).

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Large-scale Preparation of Partially Digested DNA

1. After conditions have been established in pilot experiments (see pages 9.24–9.26), digest 100 μg of high-molecular-weight DNA with the appropriate amount of restriction enzyme for the appropriate time.

   To ensure that the conditions for the large-scale digestion are as identical as possible to those used in the pilot experiment, we prefer to set up replicas of the successful pilot reaction rather than a single large-scale reaction. In addition, if sufficient eukaryotic DNA is available, we recommend using three different concentrations of restriction enzyme that straddle the optimal concentration determined in the pilot experiment.

   At the end of the digestion, analyze an aliquot of the DNA in each digestion by gel electrophoresis to make sure that the digestion has worked according to prediction. Until the results are available, store the remainder of the sample at 0°C.

   If all has gone well, select DNA in the size range appropriate for a particular bacteriophage λ or cosmid vector by sedimentation through a 10–40% sucrose density gradient as described below. Alternatively, if you plan to construct a genomic DNA library in cosmids and if the amount of DNA is limited, treat the DNA with calf intestinal alkaline phosphatase as described in Chapter 3, pages 3.38–3.39.

2. Gently extract the digested DNA twice with phenol:chloroform. Precipitate the DNA with 2 volumes of ethanol at 0°C and redissolve it in 200 μl of TE (pH 8.0).

3. Prepare a 10–40% continuous sucrose density gradient in a Beckman SW40 polyallomer tube (or its equivalent). The sucrose solutions are made in a buffer containing 10 mM Tris · Cl (pH 8.0), 10 mM NaCl, 1 mM EDTA (pH 8.0). Heat the DNA sample for 10 minutes at 68°C, cool it to 20°C, and load it onto the gradient. Centrifuge at 22,000 rpm for 22 hours at 20°C in a Beckman SW40 rotor (or its equivalent).

4. Using a 19-gauge needle, puncture the bottom of the tube and collect 350-μl fractions.

5. Mix 10 μl of every other fraction with 10 μl of water and 5 μl of gel-loading buffer I (see Appendix B). Analyze the size of the DNA in each fraction by electrophoresis through a 0.5% agarose gel, using oligomers of plasmid DNA as markers. Be sure to adjust the sucrose and salt concentrations of the markers to correspond to those of the samples.

6. Following electrophoresis, pool the gradient fractions containing DNA fragments of the desired size (e.g., 35–45 kb for construction of libraries in cosmids; 20–25 kb for construction of libraries in bacteriophage λ vectors such as EMBL3 and 4). Dialyze the pooled fractions against 2 liters of TE (pH 8.0) at 4°C for 12–16 hours, with a change of buffer after 4–6 hours. Leave space in the dialysis bag for the sample to expand two- to threefold.

   Alternatively, if the volume of the pooled sample is sufficiently small, the DNA can be precipitated with ethanol without prior dialysis after first diluting the sample with TE (pH 8.0) so that the concentration of sucrose is reduced to below 10%.

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7. Extract the dialyzed DNA several times with an equal volume of 2-butanol (see Appendix E) until the volume is reduced to about 1 ml. Add 10 M ammonium acetate to a final concentration of 2 M and precipitate the DNA with 2 volumes of ethanol at room temperature.

8. Dissolve the DNA in TE (pH 8.0) at a concentration of 300–500 μg/ml. Analyze an aliquot of the DNA (0.5 μg) by electrophoresis through a 0.5% agarose gel to check that the size distribution of the digestion products is correct.

9. To establish genomic DNA libraries, ligate the fractionated DNA to cosmid vectors or to the arms of bacteriophage λ vectors as described in Chapters 3 and 2, respectively.

10. Set up a series of trial ligations to determine the optimal ratio of vector to size-selected fragments of genomic DNA. Package an aliquot of each of the ligations, and use the resulting bacteriophages to infect the appropriate strain of *E. coli*. (See Chapters 2 and 3 for protocols.)

11. Pick 6–12 plaques (bacteriophage λ) or bacterial colonies (cosmids) and prepare a small amount of recombinant DNA from each as described in Chapter 2, for bacteriophage λ, or Chapter 3, for cosmids. Check the size of the inserts of genomic DNA by digestion with the appropriate restriction enzymes followed by electrophoresis through a 0.7% agarose gel, using markers of the appropriate size.

12. If the results are satisfactory, set up a large-scale ligation and packaging reaction and establish a genomic DNA library of the desired size. Amplify the library and store it in small aliquots as described on page 9.30 (or see Chapter 3, pages 3.44–3.53, for specific procedures for amplification and storage of cosmid libraries).

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Partial Filling of Recessed 3′ Termini of Fragments of Genomic DNA

1. Partially digest 25 μg of high-molecular-weight genomic DNA with *Sau*3AI as described on pages 9.27–9.28. The modal size of the fragments should be 20–23 kb. Purify the fragments by extraction with phenol:chloroform. Concentrate the fragments by precipitation with 2 volumes of ethanol at 0°C. Collect the fragments by centrifugation at 12,000*g* for 10 minutes at 0°C in a microfuge. Wash the pellet of nucleic acid with 70% ethanol and recentrifuge for 2 minutes. Remove the ethanol by careful aspiration, and allow the pellet to dry at room temperature. Redissolve the pellet in TE (pH 7.6) at a concentration of 1 μg/ml. Check the recovery of the DNA by analyzing a small aliquot by electrophoresis through a 0.7% agarose gel, using markers of the appropriate size.

2. In a sterile microfuge tube, mix:

partially digested genomic DNA	20 μg
10 × *Sau*3AI restriction enzyme buffer	5 μl
1 mM dATP	2 μl
1 mM dGTP	2 μl
H_2O to 46 μl	
Klenow fragment of *E. coli* DNA polymerase I (5 units/μl)	4 μl

   Incubate the reaction for 30 minutes at 37°C.

3. Purify the DNA by extraction with phenol:chloroform and chloroform.

4. After the last extraction, transfer the supernatant to a fresh tube, and add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol at 4°C. Store the sample on ice for 10 minutes, and then collect the DNA by centrifugation at 12,000*g* for 10 minutes at 0°C in a microfuge. Wash the pellet with 70% ethanol at room temperature, and recentrifuge for 2 minutes at 0°C. Remove the ethanol by careful aspiration, and allow the pellet to dry at room temperature. Redissolve the DNA in TE (pH 7.6) at a concentration of 1 μg/ml. Check the recovery of the DNA by analyzing a small aliquot by electrophoresis through a 0.7% agarose gel.

5. Set up a series of trial ligations to determine the optimal ratio of partially filled bacteriophage λ vector arms and partially filled fragments of genomic DNA. Use the ligation conditions recommended by the manufacturer of the arms or see Chapter 2, page 2.94, for ligation conditions if arms were prepared in the laboratory. Package an aliquot of each of the ligations (see Chapter 2, pages 2.95–2.107) and plate the resulting bacteriophages on the appropriate host (see Chapter 2, pages 2.61–2.62).

6. Pick 6–12 plaques and prepare a small amount of bacteriophage λ DNA from each plaque as described in Chapter 2. Check the size of the inserts of genomic DNA by digestion with the appropriate restriction enzymes followed by electrophoresis through a 0.7% agarose gel.

7. If the results are satisfactory, set up a large-scale ligation and packaging reaction and establish a genomic DNA library of the desired size. Amplify the library and store it in small aliquots as described on page 9.30.

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Amplification of a Genomic DNA Library

The library of recombinant bacteriophages may be amplified by growing a plate stock directly from the packaging mixture as described below. However, under some circumstances, it may be advisable to omit the amplification step and to screen the recombinant bacteriophages in the packaging mixture directly for those that contain the DNA sequences of interest. If, for example, the recombinant you seek grows poorly, it may be heavily underrepresented in the amplified library. Such recombinants, even those that form minute plaques, can sometimes be isolated if the amplification step is omitted. Packaging mixtures containing recombinant bacteriophages are plated out and the resulting plaques are screened directly by hybridization.

1. Mix aliquots of the packaging mixture (or concentrated bacteriophage suspension) containing 10,000–20,000 recombinant bacteriophages in a volume of 50 μl or less with 0.2 ml of plating bacteria. Incubate for 20 minutes at 37°C.

2. Mix 6.5 ml of melted top agar or agarose with each aliquot of infected bacteria and spread on a freshly poured 150-mm plate of bottom agar.

   Alternatively, as many as 450,000 bacteriophages may be mixed with 14 ml of bacteria and plated in 75 ml of top agar or agarose on 500 ml of bottom agar in a 23-cm × 33-cm glass baking dish.

3. Incubate at 37°C for a maximum of 8–10 hours. Do not allow the plaques to grow so large that they touch. This short period of growth minimizes both the chances for infection of bacteria with two different recombinants, thereby reducing the possibility of recombination between repetitive sequences carried by different recombinants with consequent "scrambling" of the library, and the opportunity for changes in the bacteriophage population that may occur because of differences in the rate of growth of different recombinants.

4. Overlay the plates with 12 ml of SM (or 150 ml of SM if baking dishes are used). Store the plates overnight at 4°C on a level surface.

5. Recover the bacteriophage suspension from each plate and transfer the suspension to a sterile polypropylene tube. Rinse the plate with 4 ml of SM. Add 0.2 ml of chloroform. Incubate for 15 minutes at room temperature with occasional shaking.

6. Remove cell and agar debris by centrifugation at 4000*g* for 5 minutes at 4°C.

7. Recover the supernatant and place in a glass tube or bottle. Add 1 or 2 drops of chloroform and store in aliquots at 4°C. The titer of the library will be stable for several years.

Analysis of Genomic DNA by Southern Hybridization

Localization of particular sequences within genomic DNA is usually accomplished by the transfer techniques described by Southern (1975). Genomic DNA is digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denatured in situ and transferred from the gel to a solid support (usually a nitrocellulose filter or nylon membrane). The relative positions of the DNA fragments are preserved during their transfer to the filter. The DNA attached to the filter is hybridized to radiolabeled DNA or RNA, and autoradiography is used to locate the positions of bands complementary to the probe. The techniques described below are suitable for analysis of restriction digests of mammalian genomic DNA. However, they can easily be adapted to accommodate restriction digests of plasmids, cosmids, and λ bacteriophages.

The amount of genomic DNA needed to generate a detectable hybridization signal depends on a number of factors, including the proportion of the genome that is complementary to the probe, the size of the probe and its specific activity, and the amount of genomic DNA transferred to the filter. Under the best conditions, the method is sufficiently sensitive to detect, in a autoradiographic exposure of several days, less than 0.1 pg of DNA complementary to a probe that has been radiolabeled with ^{32}P to high specific activity ($> 10^9$ cpm/μg; see Chapter 10). A sequence of 1000 bp that occurs only once in the mammalian genome (i.e., 1 part in 3 million) can be detected in an overnight exposure if 10 μg of genomic DNA is transferred to the filter and hybridized to a probe several hundred nucleotides in length. Because the strength of the signal is proportional to the specific activity of the probe and inversely proportional to its length, Southern hybridization reaches the limits of its sensitivity when very short probes are used. To obtain a detectable signal with oligonucleotide probes, it is therefore necessary to radiolabel oligonucleotides to the highest specific activity possible, to increase the amount of target DNA on the filter, and to expose the autoradiograph for several days.

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SEPARATION OF RESTRICTION FRAGMENTS OF MAMMALIAN GENOMIC DNA BY AGAROSE GEL ELECTROPHORESIS

1. Digest an appropriate amount of DNA with one or more restriction enzymes. For Southern analysis of mammalian genomic DNA, approximately 10 μg of DNA should be loaded into each slot of the gel when probes of standard length (>500 bp) and high specific activity ($>10^9$ cpm/μg) are used to detect single-copy sequences; 30–50 μg of DNA are needed when oligonucleotides are used as probes. Proportionately less DNA may be used when the sample contains higher concentrations of the sequences of interest.

   When handling high-molecular-weight genomic DNA, use either pipette tips that have been cut off with a sterile razor blade to enlarge the orifices or disposable wide-bore capillaries.

   The concentrations of DNA in preparations of high-molecular-weight mammalian genomic DNA are often so low that it is necessary to carry out restriction digests in relatively large volumes. After digestion is complete, the fragments of DNA are concentrated by precipitation with ethanol and are applied to the gel in a small volume of gel-loading buffer (see step 2). However, it is essential to ensure that the ethanol is removed from the DNA solution before it is loaded on the gel. If significant quantities of ethanol remain, the DNA "crawls" out of the slot of the gel. Heating the solution of redissolved DNA to 70°C in an open tube for 10 minutes is usually sufficient to drive off most of the ethanol. This treatment also disrupts base pairing between cohesive termini of restriction fragments.

   The chief problem encountered during digestion of high-molecular-weight DNA is unevenness of digestion caused by variations in the local concentrations of DNA. Clumps of DNA are relatively inaccessible to restriction enzymes and can be digested only from the outside. To ensure homogeneous dispersion of the DNA:
   a. Allow the DNA to stand at 4°C for several hours after dilution and addition of 10× restriction enzyme buffer.
   b. *Gently* stir the DNA solution from time to time using a sealed glass capillary.
   c. After addition of the restriction enzyme, *gently* stir the solution for 2–3 minutes at 4°C before warming the reaction to the appropriate temperature.
   d. After digestion for 15–30 minutes, add a second aliquot of restriction enzyme and stir the reaction as described above.

   It is important to include controls to show whether digestion with the restriction enzyme(s) is complete and whether transfer and hybridization of the DNA has worked efficiently. This can be accomplished by setting up a series of digests containing mammalian DNA and a very small amount of a plasmid carrying a sequence complementary to the probe (e.g., 10 μg of mammalian DNA and 10^{-5}, 10^{-6}, and 10^{-7} μg of plasmid). During digestion, the plasmid will be cleaved into a series of bands that will be invisible when the gel is examined by staining with ethidium bromide. However, fragments of the correct size should be detected by subsequent hybridization to the probe. These controls should be loaded into wells that lie toward one side of the gel, well away from the test samples of mammalian DNA. This reduces the chances of accidental contamination and minimizes the possibility that the hybridization from the controls will obscure the signal from the test samples.

2. At the end of the digestion, add the appropriate amount of gel-loading buffer (see Appendix B) and separate the fragments of DNA by electrophoresis through an agarose gel (for genomic DNA, a 0.7% gel cast in 0.5× TBE containing ethidium bromide [0.5 μg/ml] may be used; see Chapter 6, pages 6.9–6.13).

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

It is important to include controls containing DNAs of known size that can serve as molecular-weight standards (e.g., bacteriophage λ DNA cleaved with *Hin*dIII). These marker DNAs are usually run in the two outside wells of the gel. Sufficient marker DNA to be detectable by staining with ethidium bromide (~200 ng) should be applied to the gel.

Occasionally, problems arise during loading of the gel because the DNA solution will not sink to the bottom of the well. This occurs when very-high-molecular-weight DNA is present at the end of the digest, for example, when the digest is incomplete or when mammalian DNA has been digested with enzymes such as *Not*I that generate very large fragments of DNA. To minimize the problem, make sure that the DNA is homogeneously dispersed and load the samples very slowly into the wells of the gel. After loading, allow the gel to stand for a few minutes so that the DNA can diffuse evenly throughout the wells.

If the DNA has been stored at 4°C, it should be heated to 56°C for 2–3 minutes before it is applied to the gel. This disrupts any base pairing that may have occurred between protruding cohesive termini.

The gel should be run slowly (<1 V/cm) to prevent smearing of the fragments of DNA. Ethidium bromide (0.5 μg/ml) is usually included in both the gel and the electrophoresis buffer. However, some workers believe that the presence of the dye increases the tendency of bands of high-molecular-weight DNA to "smile" during electrophoresis. They therefore carry out electrophoresis in the absence of ethidium bromide and stain the gel later.

3. After electrophoresis is completed, photograph the gel as described in Chapter 6, page 6.19. Place a transparent ruler alongside the gel so that the distance that any band of DNA has migrated can be read directly from the photographic image.

If desired, the gel may be stored at this stage before the DNA is denatured and transferred to the filter. Wrap the gel in Saran Wrap and store it on a flat surface at 4°C. Because the bands of DNA diffuse during storage, the gel should not be put aside for more than 1 day before being processed.

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TRANSFER OF DNA FROM AGAROSE GELS TO SOLID SUPPORTS

There are three methods to transfer fragments of DNA from agarose gels to solid supports (nitrocellulose filters or nylon membranes):

1. *Capillary transfer.* In the capillary transfer method (Southern 1975), DNA fragments are carried from the gel in a flow of liquid and deposited on the surface of the solid support. The liquid is drawn through the gel by capillary action that is established and maintained by a stack of dry, absorbent paper towels (Figure 9.3). The rate of transfer of the DNA depends on the size of the DNA fragments and the concentration of agarose in the gel. Small fragments of DNA (<1 kb) are transferred almost quantitatively from a 0.7% agarose gel within 1 hour; larger fragments are transferred more slowly and less efficiently. For example, capillary transfer of DNAs greater than 15 kb in length requires at least 18 hours, and even then the transfer is not complete. The efficiency of transfer of large DNA fragments is determined by the fraction of molecules that escape from the gel before it becomes dehydrated. As elution proceeds, fluid is drawn not only from the reservoir, but also from the interstices of the gel itself. This reduces the gel to a rubbery substance through which DNA molecules cannot pass. This problem of dehydration can be partially alleviated by partial hydrolysis of the DNA prior to capillary transfer (Wahl et al. 1979; Meinkoth and Wahl 1984). The DNA in the gel is exposed to weak acid (which results in partial depurination), followed by strong base (which hydrolyzes the phosphodiester backbone at the sites of depurination). The resulting fragments of DNA (~1 kb in length) can then be transferred rapidly from the gel with high efficiency. However, it is important not to let the depurination reaction proceed too far; otherwise, the DNA is cleaved into small fragments that are too short to bind efficiently to the solid support. Depurination/hydrolysis can also cause the bands of the final autoradiograph to assume a "fuzzy" appear-

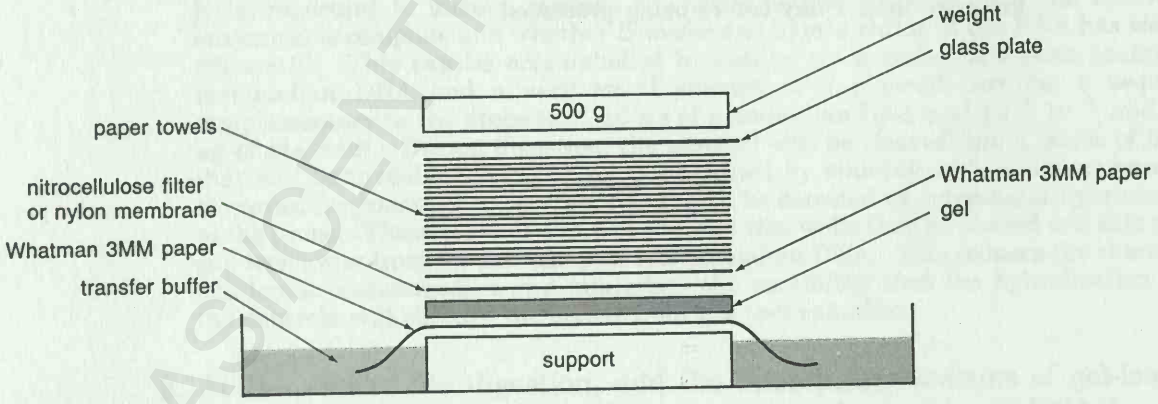

FIGURE 9.3
Capillary transfer of DNA from agarose gels. Buffer is drawn from a reservoir and passes through the gel into a stack of paper towels. The DNA is eluted from the gel by the moving stream of buffer and is deposited on a nitrocellulose filter or nylon membrane. A weight applied to the top of the paper towels helps to ensure a tight connection between the layers of material used in the transfer system.

ance, presumably because of increased diffusion of DNA during transfer. Depurination/hydrolysis is therefore recommended only when it is known ahead of time that the target DNA fragments exceed 15 kb in length.

When the target DNA fragments are present in high concentration (e.g., in restriction digests of plasmids or bacteriophage λ recombinants), the capillary method can be used to transfer DNA simultaneously and rapidly from a single gel to two nitrocellulose filters or nylon membranes (Smith and Summers 1980) (Figure 9.4). The only source of transfer buffer is the liquid trapped in the gel itself, and the efficiency of transfer is therefore relatively poor. This method is not recommended when high sensitivity is required (e.g., detection of single-copy sequences in mammalian DNA), but it is perfectly adequate for Southern analysis of recombinant plasmids and bacteriophage λ DNAs.

2. *Electrophoretic transfer.* This method is not practical when nitrocellulose is used as the solid support because of the high ionic strengths of the buffers that are required to bind nucleic acids to these filters. These buffers conduct electric current very efficiently, and it is necessary to use large volumes to ensure that the buffering power of the system does not become depleted by electrolysis. In addition, extensive external cooling is required to overcome the effects of ohmic heating (Arnheim and Southern 1977). Until recently, therefore, electrophoretic transfer was carried out with supports such as diazobenzyloxymethyl (DBM)- or *o*-aminophenylthioether (APT)-cellulose, which bind nucleic acids efficiently at low ionic strength (see, e.g., Bittner et al. 1980; Stellwag and Dahlberg 1980). Although these types of solid supports are no longer in common use, electrophoretic transfer has undergone a recent resurgence with the advent of charged nylon membranes. Nucleic acids as small as 50 bp will bind to these membranes in buffers of very low ionic strength (Reed and Mann 1985).

Although single-stranded DNA and RNA can be transferred directly, fragments of double-stranded DNA must first be denatured in situ as described on page 9.38. The gel is then neutralized and soaked in

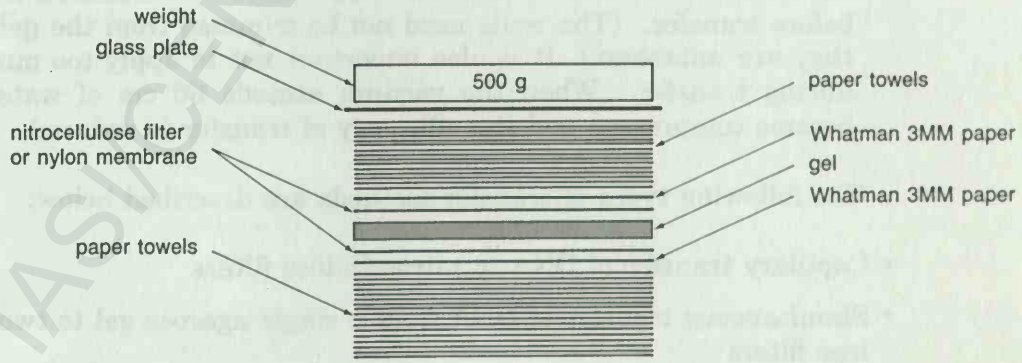

FIGURE 9.4
Capillary transfer of DNA from agarose gels to two solid supports simultaneously.

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electrophoresis buffer (1 × TBE; see Appendix B) before being mounted between porous pads aligned between parallel electrodes in a large tank of buffer. The time required for complete transfer depends on the size of the fragments of DNA, the porosity of the gel, and the strength of the applied field. However, because even high-molecular-weight nucleic acids migrate relatively rapidly from the gel, depurination/hydrolysis is unnecessary and transfer is generally complete within 2–3 hours. Because electrophoretic transfer requires comparatively large electric currents, it is often difficult to maintain the electrophoresis buffer at a temperature compatible with efficient transfer of DNA. Many commercially available electrophoretic transfer machines are equipped with cooling devices, but others are effective only when used in a coldroom. Because of these and other problems, we recommend that electrophoretic transfer be used only when transfer by capillary action or under vacuum is inefficient, for example, when it is necessary to analyze fragments of DNA separated by electrophoresis through polyacrylamide gels (Stellwag and Dahlberg 1980; Church and Gilbert 1984).

3. *Vacuum transfer.* DNA and RNA can be transferred rapidly and quantitatively from gels under vacuum. Several vacuum transfer devices are now available commercially in which the gel is placed in contact with a nitrocellulose filter or nylon membrane supported on a porous screen over a vacuum chamber. Buffer, drawn from an upper reservoir, elutes nucleic acids from the gel and deposits them on the filter or membrane.

   Vacuum transfer is more efficient than capillary transfer and is extremely rapid; DNAs that have been partially depurinated and denatured with alkali are quantitatively transferred within 30 minutes from gels of normal thickness (4–5 mm) and normal agarose concentration (<1%). If carried out carefully, vacuum transfer can result in a two- to threefold enhancement of the hybridization signal obtained from Southern transfers (Medveczky et al. 1987; Olszewska and Jones 1988).

   All of the commercially available apparatuses work well as long as care is taken to ensure that the vacuum is applied evenly over the entire surface of the gel. Special care should be taken with the wells of horizontal agarose gels, which tend to break during preparation of the gel for transfer. If this occurs, the wells should be trimmed from the gel before transfer. (The wells need not be trimmed from the gel as long as they are unbroken.) It is also important not to apply too much vacuum during transfer. When the vacuum exceeds 60 cm of water, the gels become compressed and the efficiency of transfer is reduced.

The following types of transfer methods are described below:

- Capillary transfer of DNA to nitrocellulose filters
- Simultaneous transfer of DNA from a single agarose gel to two nitrocellulose filters
- Capillary transfer of DNA to nylon membranes under both neutral and alkaline conditions

We have not given detailed descriptions of the methods used for electrophoretic transfer and vacuum transfer. We recommend that these techniques be carried out according to the instructions provided by the manufacturer of the apparatus that is used.

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CAPILLARY TRANSFER OF DNA TO NITROCELLULOSE FILTERS

1. After electrophoresis, transfer the gel to a glass baking dish and trim away any unused areas of the gel with a razor blade. Cut off the bottom left-hand corner of the gel; this serves to orient the gel during the succeeding operations.

2. Denature the DNA by soaking the gel for 45 minutes in several volumes of 1.5 M NaCl, 0.5 N NaOH with constant, *gentle* agitation (e.g., on a rotary platform).

   If the gel floats to the surface of the liquid, weigh it down with several pasteur pipettes.

   If the fragments of interest are larger than approximately 15 kb, transfer may be improved by nicking the DNA by brief depurination prior to denaturation with base. After step 1, soak the gel for 10 minutes in several volumes of 0.2 N HCl and then rinse briefly with deionized water.

3. Rinse the gel briefly in deionized water, and then neutralize it by soaking for 30 minutes in several volumes of a solution of 1 M Tris (pH 7.4), 1.5 M NaCl at room temperature with constant, gentle agitation. Change the neutralization solution and continue soaking the gel for a further 15 minutes.

4. While the gel is in the neutralization solution, wrap a piece of Whatman 3MM paper around a piece of Plexiglas or a stack of glass plates to form a support that is longer and wider than the gel. Place the wrapped support inside a large baking dish. Fill the dish with transfer buffer (10 × SSC or 10 × SSPE) until the level of the liquid reaches almost to the top of the support. When the 3MM paper on the top of the support is thoroughly wet, smooth out all air bubbles with a glass rod.

   20 × SSC or 20 × SSPE can also be used as the transfer buffer. The binding of DNA to nitrocellulose depends on the ionic strength of the transfer buffer (Gillespie and Spiegelman 1965). The smaller the fragments of DNA, the higher the ionic strength required for their efficient retention on the nitrocellulose filter. For Southern transfer of fragments of DNA less than 500 nucleotides in length, 20 × SSC or 20 × SSPE should be used (Nagamine et al. 1980). Alternatively, nylon membranes, which bind small DNA fragments more efficiently than nitrocellulose filters, may be used (see pages 9.42–9.46).

   The efficiency with which small fragments of DNA are retained by nitrocellulose also depends on the pore size of the filter. Fragments less than 300 nucleotides in length are not retained by standard nitrocellulose filters, whose pore size is 0.45 micron. The efficiency of transfer can be improved if nitrocellulose filters with a pore size of 0.2 micron are used.

5. Using a fresh scalpel or a paper cutter, cut a piece of nitrocellulose filter (Schleicher and Schuell BA85 or equivalent) about 1 mm larger than the gel in both dimensions. Use gloves and blunt-ended forceps (e.g., Millipore forceps) to handle the filter. A nitrocellulose filter that has been touched by greasy hands will not wet!

6. Float the nitrocellulose filter on the surface of a dish of deionized water until it wets completely from beneath, and then immerse the filter in transfer buffer for at least 5 minutes. Using a clean scalpel blade, cut a corner from the nitrocellulose filter to match the corner cut from the gel.

   The rate at which different batches of nitrocellulose filters wet varies enormously. If the filter is not saturated after floating for several minutes on water, it should be replaced with a new filter, since the transfer of DNA to an unevenly wetted filter is unreliable. The original filter should not be discarded but should be autoclaved for 5 minutes between pieces of 3MM paper saturated with 2 × SSC. This usually results in complete wetting of the filter. The autoclaved filter, sandwiched between the autoclaved 3MM papers saturated with 2 × SSC, may be stored at 4°C in a sealed plastic bag until it is needed.

7. Remove the gel from the neutralization solution and invert it so that its underside is now uppermost. Place the inverted gel on the support so that it is centered on the wet 3MM papers. Make sure that there are no air bubbles between the 3MM paper and the gel.

8. Surround, but do not cover, the gel with Saran Wrap or Parafilm. This serves as a barrier to prevent liquid from flowing directly from the reservoir to paper towels placed on top of the gel. If these towels are not precisely stacked, they tend to droop over the edge of the gel and may touch the support. This type of short-circuiting is a major cause of inefficient transfer of DNA from the gel to the filter.

9. Place the wet nitrocellulose filter on top of the gel so that the cut corners are aligned. One edge of the filter should extend just over the edge of the line of slots at the top of the gel. Do not move the filter once it has been applied to the surface of the gel. Make sure that there are no air bubbles between the filter and the gel.

10. Wet two pieces of 3MM paper (cut to exactly the same size as the gel) in 2 × SSC and place them on top of the wet nitrocellulose filter. Smooth out any air bubbles with a glass rod.

11. Cut a stack of paper towels (5–8 cm high) just smaller than the 3MM papers. Place the towels on the 3MM papers. Put a glass plate on top of the stack and weigh it down with a 500-g weight (see Figure 9.3). The objective is to set up a flow of liquid from the reservoir through the gel and the nitrocellulose filter, so that fragments of denatured DNA are eluted from the gel and are deposited on the nitrocellulose filter.

12. Allow the transfer of DNA to proceed for 8–24 hours. As the paper towels become wet, they should be replaced.

13. Remove the paper towels and the 3MM papers above the gel. Turn over the gel and the nitrocellulose filter and lay them, gel side up, on a dry sheet of 3MM paper. Mark the positions of the gel slots on the filter with a very-soft-lead pencil or a ballpoint pen.

14. Peel the gel from the filter and discard it. Soak the filter in 6 × SSC for 5 minutes at room temperature. This removes any pieces of agarose sticking to the filter.

To assess the efficiency of transfer of DNA, the gel may be stained for 45 minutes in a solution of ethidium bromide (0.5 μg/ml in water) and examined by ultraviolet illumination. Note that the intensity of fluorescence will be quite low because the DNA remaining in the gel has been denatured.

Cautions: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

15. Remove the filter from the 6 × SSC and allow excess fluid to drain away. Place the filter flat on a paper towel to dry for at least 30 minutes at room temperature.

16. Sandwich the filter between two sheets of dry 3MM paper. Fix the DNA to the filter by baking for 30 minutes to 2 hours at 80°C in a vacuum oven.

Overbaking can cause the filter to become brittle. If the gel was not completely neutralized before the DNA was transferred, the filter will turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

17. Hybridize the DNA immobilized on the filter to a ^{32}P-labeled probe as described on pages 9.52–9.55.

Any filters not used immediately in hybridization reactions should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

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SIMULTANEOUS TRANSFER OF DNA FROM A SINGLE AGAROSE GEL TO TWO NITROCELLULOSE FILTERS

DNA can be transferred simultaneously from a single agarose gel to two nitrocellulose filters as described by Smith and Summers (1980) (see Figure 9.4).

1. After electrophoresis, prepare the gel for transfer as described in steps 1–3 on page 9.38.

2. After neutralization, soak the gel for 30 minutes in 10× SSC. Cut the bottom left-hand corner from the gel; this provides a convenient way to orient the gel and the nitrocellulose filters.

3. While the gel is soaking, cut and wet two pieces of nitrocellulose (0.45-micron pore size), first in water and then in 10× SSC. Cut one corner from each filter.

   To retain small fragments of DNA (< 300 nucleotides), use nitrocellulose filters with a small pore size (0.2 micron).

4. Lay one of the nitrocellulose filters on a piece of dampened 3MM paper. Lay the gel on top of the filter, aligning the cut corner of the gel with the cut corner of the filter. Without delay, cover the gel with the second nitrocellulose filter, followed by another piece of dampened 3MM paper. Make sure that there are no air bubbles between the filters and the gel.

5. Transfer the entire sandwich of 3MM papers, nitrocellulose filters, and gel onto a stack of paper towels. Cover the sandwich with a second stack of paper towels. Put a glass plate on top of the entire stack and weigh it down with a 500-g weight.

6. After 2 hours, remove the nitrocellulose filters, mark the approximate positions of the gel slots with a very-soft-lead pencil or a ballpoint pen, and process them for hybridization as described on page 9.40 (step 14 onward).

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Transfer of DNA from Agarose Gels to Nylon Membranes

For almost 20 years, immobilization and hybridization of denatured DNA was carried out exclusively with nitrocellulose—first with powder (Hall and Spiegelman 1961) and then with sheets (Nygaard and Hall 1963; Gillespie and Spiegelman 1965). Despite its evident success, nitrocellulose is not an ideal matrix for solid-phase hybridization. First, nucleic acids are attached by hydrophobic rather than covalent interactions and are released slowly from the matrix during hybridization and washing at high temperatures. Second, nitrocellulose filters become brittle when dry and cannot survive more than one or two cycles of hybridization and washing. In recent years, therefore, a number of alternative supporting matrices have been explored. These include DBM-cellulose (Goldberg et al. 1979; Wahl et al. 1979) and APT-cellulose (Seed 1982a,b), both of which bind DNA and RNA covalently. Nucleic acids attached to these supports do not leach during hybridization or washing at high temperature and can therefore be hybridized to several probes sequentially. However, activated derivatives of cellulose are laborious to synthesize and do not stand up to the rigors of hybridization much better than nitrocellulose itself. This problem has been solved by the introduction of various types of nylon membranes that bind nucleic acids irreversibly and are far more durable than nitrocellulose filters. Immobilized nucleic acids can therefore be hybridized sequentially to several different probes without damaging the membrane. Furthermore, because nucleic acids can be immobilized on nylon in buffers of low ionic strength, transfer of nucleic acids from gels to nylon membranes can be carried out electrophoretically. This can be useful when capillary or vacuum transfer of DNA is inefficient, for example, when fragments of DNA are transferred from polyacrylamide gels. In addition, large membranes can be handled without fear that they will split, tear, or curl. The sole disadvantage of nylon membranes is a tendency to give increased levels of background hybridization, especially with RNA probes. In many cases, this problem can be overcome by using increased amounts of blocking agents in the prehybridization and hybridization steps.

Two basic types of nylon membranes are available commercially—unmodified nylon and charge-modified nylon. Although both types of membranes can bind nucleic acids, the latter is preferred for transfer and hybridization, since its positively charged surface has a greater capacity for binding nucleic acids. Many different types of charged nylon membranes are available that vary in the type of charge, the method used to apply it, and the density of the nylon mesh. Both double- and single-stranded nucleic acids may be applied to nylon membranes in a wide range of solvents (retention is quantitative in solvents as diverse as water, 0.25 N HCl, and 0.4 N NaOH). However, each manufacturer provides specific instructions for the transfer of nucleic acids to their particular type of charged nylon membrane. These instructions should be followed exactly, since they presumably have been shown to yield the best results. (For practical information about the use of nylon membranes, see Reed and Mann [1985].) The general protocol given below works well with most types of positively charged nylon membranes but may not be optimal for any particular brand.

Nylon membranes must be treated to immobilize the DNA after it has been transferred. The DNA becomes fixed to the nylon membrane if it is thorough-

ly dried (e.g., under vacuum) or if it is exposed to low doses of ultraviolet irradiation (254 nm) after transfer of the nucleic acid. The latter method, although a nuisance to set up, is preferred since it is much quicker and the nucleic acid becomes covalently attached to the membrane. In addition, there are reports that DNA transferred to nylon membranes in buffers of high ionic strength and immobilized with ultraviolet irradiation yields greatly enhanced hybridization signals (Khandjian 1987).

Hybridization of radiolabeled probes to nucleic acids immobilized on nylon membranes is carried out essentially as described on pages 9.52–9.55 for nitrocellulose filters. Either formamide or aqueous solvents may be used, and background binding of the probe may be suppressed by including either 1% SDS, Denhardt's reagent, or 0.05 × BLOTTO.

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CAPILLARY TRANSFER OF DNA TO NYLON MEMBRANES UNDER NEUTRAL CONDITIONS

1. Fractionate the DNA by gel electrophoresis, and process the gel as described in steps 1–3 on page 9.38.

2. While the gel is in the neutralization solution, prepare the nylon membrane as follows:

   a. Using a fresh scalpel or a paper cutter, cut a piece of membrane about 1 mm larger than the gel in both dimensions. Use gloves and blunt-ended forceps (e.g., Millipore forceps) to handle the membrane.

   b. Float the membrane on the surface of a dish of deionized water until it wets completely from beneath, and then immerse the membrane in transfer buffer (10 × SSC or 10 × SSPE) for at least 5 minutes. Using a clean scalpel blade, cut a corner from the membrane to match the corner cut from the gel.

3. Transfer the denatured DNA from the gel to the membrane by capillary action as described in steps 7–15 on pages 9.39–9.40.

4. To fix the DNA to the membrane either

   - place the dried membrane between two pieces of 3MM paper, and bake the membrane for 30 minutes to 2 hours at 80°C in a vacuum or conventional oven, or
   - expose the side of the membrane carrying the DNA to a source of ultraviolet irradiation (254 nm).

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

   Immobilization of nucleic acids by ultraviolet irradiation can greatly enhance the hybridization signal obtained with some brands of positively charged nylon membranes (Khandjian 1987). However, for maximum effect, it is important to make sure that the membrane is not overirradiated. The aim is to form cross-links between a small fraction of the thymine residues in the DNA and positively charged amine groups on the surface of the membrane (Church and Gilbert 1984). Overirradiation results in the covalent attachment of a high proportion of the thymines, with consequent decrease in hybridization signal. The side of the membrane carrying the DNA should face the ultraviolet light source. Most manufacturers advise that damp membranes should be exposed to a total of 1.5 J/sq. cm and that dry membranes should be exposed to 0.15 J/sq. cm. However, we recommend carrying out a series of preliminary experiments to determine empirically the amount of irradiation required to produce the maximum hybridization signal.

   If the membrane is not to be used immediately in hybridization experiments, it should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

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CAPILLARY TRANSFER OF DNA TO NYLON MEMBRANES UNDER ALKALINE CONDITIONS

The ability of positively charged nylon membranes to bind denatured DNA under alkaline conditions has advantages in some circumstances, for example, when using probes that are complementary to inverted repeat sequences. However, the level of background hybridization is almost always significantly higher when the membrane has been exposed to high concentrations of alkali for extended periods of time. This problem can often be overcome by increasing the concentration of blocking agents in the hybridization step.

1. After electrophoresis, transfer the gel to a glass baking dish and trim away any unused areas of the gel with a razor blade. Cut off the bottom left-hand corner of the gel; this serves to orient the gel during the succeeding operations.

2. Denature the DNA by soaking the gel for 15 minutes in denaturation/transfer solution (0.4 N NaOH, 1 M NaCl) with constant, *gentle* agitation (e.g., on a rotary platform). Change the solution and continue to soak the gel for a further 20 minutes with gentle agitation.

   If the gel floats to the surface of the liquid, weigh it down with several pasteur pipettes.

   If the fragments of interest are larger than approximately 15 kb, transfer may be improved by nicking the DNA by brief depurination prior to denaturation with base. After step 1, soak the gel for 10 minutes in several volumes of 0.2 N HCl and then rinse briefly with deionized water.

3. While the gel is soaking in the denaturation/transfer solution, prepare the nylon membrane as follows:

   a. Using a fresh scalpel or a paper cutter, cut a piece of membrane about 1 mm larger than the gel in both dimensions. Use gloves and blunt-ended forceps (e.g., Millipore forceps) to handle the membrane.

   b. Float the membrane on the surface of a dish of deionized water until it wets completely from beneath, and then immerse the membrane in denaturation/transfer solution for at least 5 minutes. Using a clean scalpel blade, cut a corner from the membrane to match the corner cut from the gel.

4. Transfer the denatured DNA from the gel to the membrane by capillary action as described in steps 7–13 on page 9.39.

5. Peel the gel from the membrane and discard it. Soak the membrane in 0.5 M Tris · Cl (pH 7.2), 1 M NaCl for 15 minutes at room temperature. This neutralizes the membrane and removes any pieces of agarose sticking to it.

   To assess the efficiency of transfer of DNA, the gel may be stained for 45 minutes in a solution of ethidium bromide (0.5 μg/ml in 0.1 M Tris · Cl [pH 7.2]) and examined by ultraviolet illumination. Note that the intensity of fluorescence will be quite low because the DNA remaining in the gel has been denatured.

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Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

6. Remove the membrane from the neutralizing solution and allow excess fluid to drain away. Place the membrane flat on a paper towel to dry for at least 30 minutes at room temperature.

7. To fix the DNA to the membrane either

   - place the dried membrane between two pieces of 3MM paper, and bake the membrane for 30 minutes to 2 hours at 80°C in a vacuum or conventional oven, or
   - expose the side of the membrane carrying the DNA to a source of ultraviolet irradiation (254 nm).

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

   Immobilization of nucleic acids by ultraviolet irradiation can greatly enhance the hybridization signal obtained with some brands of positively charged nylon membranes (Khandjian 1987). However, for maximum effect, it is important to make sure that the membrane is not overirradiated. The aim is to form cross-links between a small fraction of the thymine residues in the DNA and positively charged amine groups on the surface of the membrane (Church and Gilbert 1984). Overirradiation results in the covalent attachment of a high proportion of the thymines, with consequent decrease in hybridization signal. The side of the membrane carrying the DNA should face the ultraviolet light source. Most manufacturers advise that damp membranes should be exposed to a total of 1.5 J/sq. cm and that dry membranes should be exposed to 0.15 J/sq. cm. However, we recommend carrying out a series of preliminary experiments to determine empirically the amount of irradiation required to produce the maximum hybridization signal.

If the membrane is not to be used immediately in hybridization experiments, it should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

When hybridizing membranes that have been exposed to alkali, use the higher concentrations of blocking agents described on page 9.48, point 5.

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HYBRIDIZATION OF RADIOLABELED PROBES TO IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or polyethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.
2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.
3. Continual movement of the probe solution across the filter is unnecessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneously, agitation is advisable to prevent the filters from adhering to one another.
4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

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immobilized nucleic acid and its availability for hybridization are unknown. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1–3 \times C_o t_{1/2}$. In 10 ml of hybridization solution, 1 μg of a probe of 5-kb complexity will reach $C_o t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_o t_{1/2}$$

where x = the weight of the probe added (in micrograms), y = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and z = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_o t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by prehybridizing filters with a blocking agent consisting of 5 × Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization [1975], Benton/Davis hybridization [1977], Southern hybridization [1975] of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk (0.05 × BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.

6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Agent	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at −20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 μg/ml denatured, fragmented, salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia) 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Pentex Fraction V), and H_2O to 500 ml.

BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% non-fat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into hybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization

Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 μg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 μg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The solution is adjusted to 0.1 M NaCl and extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The OD^{260} of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at −20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 μg/ml in hybridization solutions.

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6× SSC or 6× SSPE) at a temperature that is 20–25°C below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6× SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

11. *Useful facts*:

    a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%\,G + C) - 0.63(\%\,\text{formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.

This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[Na^+]$.
- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257}. The "irreversible" T_m, which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation.

Similar equations have been derived for:

i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\%G + C) + 11.8(\%G + C)^2 - 0.35(\%\ \text{formamide}) - (820/l)$$

ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\%G + C) + 11.8(\%G + C)^2 - 0.50(\%\ \text{formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

b. The T_m of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

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Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Nitrocellulose Filters or Nylon Membranes

Although the method given below deals with RNA or DNA immobilized on nitrocellulose filters, only slight modifications are required to adapt the procedure to nylon membranes. These modifications are noted at the appropriate places in the text.

1. Prepare the prehybridization solution appropriate for the task at hand. Approximately 0.2 ml of prehybridization solution will be required for each square centimeter of nitrocellulose filter or nylon membrane.

   The prehybridization solution should be filtered through a 0.45-micron disposable cellulose acetate filter (Schleicher and Schuell Uniflow syringe filter No. 57240 or equivalent).

Prehybridization solutions

For detection of low-abundance sequences:

Either
6 × SSC (or 6 × SSPE)
5 × Denhardt's reagent
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA

or
6 × SSC (or 6 × SSPE)
5 × Denhardt's reagent
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide

For preparation of Denhardt's reagent and denatured, fragmented salmon sperm DNA, see Table 9.1.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

For detection of moderate- or high-abundance sequences:

Either
6 × SSC (or 6 × SSPE)
0.05 × BLOTTO

or
6 × SSC (or 6 × SSPE)
0.05 × BLOTTO
50% formamide

For preparation of BLOTTO, see Table 9.1.

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When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A)$^+$ RNA at a concentration of 1 μg/ml may be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

2. Float the nitrocellulose filter or nylon membrane containing the target DNA on the surface of a tray of 6× SSC (or 6× SSPE) until it becomes thoroughly wetted from beneath. Submerge the filter for 2 minutes.

3. Slip the wet filter into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent). Add 0.2 ml of prehybridization solution for each square centimeter of nitrocellulose filter or nylon membrane.

   Squeeze as much air as possible from the bag. Seal the open end of the bag with the heat sealer. Incubate the bag for 1–2 hours submerged at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide).

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

4. If the radiolabeled probe is double-stranded, denature it by heating for 5 minutes at 100°C. Single-stranded probe need not be denatured. Chill the probe rapidly in ice water.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

5. Working quickly, remove the bag containing the filter from the water bath. Open the bag by cutting off one corner with scissors. Add the denatured probe to the prehybridization solution, and then squeeze as much air as possible from the bag. Reseal the bag with the heat sealer so that as few bubbles as possible are trapped in the bag. To avoid radioactive contamination of the water bath, the resealed bag should be sealed inside a second, noncontaminated bag.

   When using nylon membranes, the prehybridization solution should be *completely* removed from the bag and immediately replaced with hybridization solution. The probe is then added and the bag is resealed.

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Hybridization solution for nylon membranes

6 × SSC (or 6 × SSPE)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide (if hybridization is to be carried out at 42°C)

6. Incubate the bag submerged in a water bath set at the appropriate temperature for the required period of hybridization.

7. Wearing gloves, remove the bag from the water bath and immediately cut off one corner. Pour out the hybridization solution into a container suitable for disposal, and then cut the bag along the length of three sides. Remove the filter and immediately submerge it in a tray containing several hundred milliliters of 2 × SSC and 0.5% SDS at room temperature.

   Important: Do not allow the filter to dry out at any stage during the washing procedure.

8. After 5 minutes, transfer the filter to a fresh tray containing several hundred milliliters of 2 × SSC and 0.1% SDS and incubate for 15 minutes at room temperature with occasional gentle agitation.

   If short oligonucleotides are used as probes, washing should be carried out only for brief periods (1–2 minutes) at the appropriate temperature. For a discussion of the stability of hybrids involving oligonucleotides, see Chapter 11.

9. Transfer the filter to a flat-bottom plastic box containing several hundred milliliters of fresh 0.1 × SSC and 0.5% SDS. Incubate the filter for 30 minutes to 1 hour at 37°C with gentle agitation.

10. Replace the solution with fresh 0.1 × SSC and 0.5% SDS, and transfer the box to a water bath set at 68°C for an equal period of time. Monitor the amount of radioactivity on the filter using a hand-held minimonitor. The parts of the filter that do not contain DNA should not emit a detectable signal. You should not expect to pick up a signal on the minimonitor from filters containing mammalian DNA that has been hybridized to single-copy probes.

11. Briefly wash the filter with 0.1 × SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

12. Place the damp filter on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filter. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

    Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot

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(>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

13. Cover the filter with a second sheet of Saran Wrap, and expose the filter to X-ray film (Kodak XAR-2 or equivalent) to obtain an autoradiographic image (see Appendix E). The exposure time should be determined empirically. However, single-copy sequences in mammalian genomic DNA can usually be detected after 16–24 hours of exposure at −70°C with an intensifying screen.

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Hybridization of Radiolabeled Oligonucleotides to Genomic DNA

Oligonucleotide probes as short as 17 nucleotides in length may be used to detect single-copy sequences in restriction digests of eukaryotic genomic DNA that have been transferred to solid supports. As discussed in Chapter 11, hybrids of this length are stable enough to be detected in practice only if they are perfectly matched. Duplexes with a single base-pair mismatch are significantly less stable and dissociate at a lower temperature than their perfectly matched counterparts (Wallace et al. 1979; Ikuta et al. 1987). It has therefore been possible to use oligonucleotides of defined sequence to probe fetal DNA for the presence of specific point mutations that cause conditions such as sickle-cell anemia (Conner et al. 1983), certain thalassemias (Orkin et al. 1983; Pirastu et al. 1983), and α_1-antitrypsin deficiency (Kidd et al. 1983); to screen DNA extracted from tumor cells for mutations in oncogenes (Bos et al. 1984, 1985, 1987; Forrester et al. 1987; Rodenhuis et al. 1987); and to analyze highly polymorphic loci, for example, the major histocompatibility complex class I genes (Geliebter et al. 1986).

The methods used when hybridizing with oligonucleotide probes are similar to those described earlier in this chapter. However, attention should be paid to the following points:

1. Because of the small size of the target sequence, a minimum of 30 μg of mammalian genomic DNA should be applied to each lane of the agarose gel.
2. The sequences of oligonucleotides used as probes should be long enough to be unique within the target genome (17 nucleotides for the mammalian genome) and short enough to allow the detection of mismatches under the conditions of hybridization used. Typically, oligonucleotides used for screening mammalian genomic DNA are 19–21 nucleotides in length.
3. When used to detect point mutations, oligonucleotides are used in pairs; one member of the pair is perfectly homologous to the mutated gene sequence and the other is homologous to the wild-type sequence. Usually, the members of the pair differ in sequence by only one nucleotide. Before embarking on an analysis of genomic DNA with these probes, it is essential to establish hybridization and washing conditions using cloned fragments of DNA of known sequence that are homologous to each member of the pair of oligonucleotides. These methods are discussed in detail in Chapter 11. Reconstruction experiments, in which known amounts of the control DNAs are added to a large excess of genomic DNA (at least 30 μg), are then used to test the sensitivity of the system.
4. Oligonucleotides are radiolabeled by [γ-^{32}P]ATP and bacteriophage T4 polynucleotide kinase (see Chapter 11). These probes tend to hybridize nonspecifically to high-molecular-weight DNA immobilized on nitrocellulose filters or nylon membranes, producing a smear toward the top of the autoradiograph. It is therefore important to choose a restriction enzyme (or a combination of restriction enzymes) that yields a hybridizing fragment whose size is not greater than 5 kb.

5. After electrophoresis, the fragments of genomic DNA may be transferred to a solid support by the conventional Southern transfer technique or immobilized within the agarose gel itself by dehydration (Studencki and Wallace 1984). Although DNA immobilized within the gel appears to give somewhat stronger hybridization signals than DNA attached to a solid support, it cannot be hybridized sequentially to many different probes. This is a severe disadvantage when the amount of genomic DNA is limited (as is often the case in prenatal diagnosis, for example). We therefore recommend that the genomic DNA be transferred to a nylon membrane such as Nytran (Schleicher and Schuell) or GeneScreen (du Pont).

6. Wherever possible, negative and positive hybridization controls should be included in each gel.

7. Oligonucleotides may also be used to detect rare transcripts in northern blots that contain 30 μg of total cellular RNA (Zeff et al. 1986) or 5 μg of poly(A)$^+$ RNA (Geliebter et al. 1986).

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Removal of Radiolabeled Probes from Nitrocellulose Filters and Nylon Membranes

Probes become irreversibly bound if nitrocellulose filters and nylon membranes are allowed to dry. Therefore, every effort should be made to ensure that the solid supports remain wet at all stages during hybridization, washing, and exposure to X-ray film.

REMOVING PROBES FROM NITROCELLULOSE FILTERS

1. Heat several hundred milliliters of 0.05 × SSC, 0.01 M EDTA (pH 8.0) to boiling. Remove the fluid from the heat and add SDS to a final concentration of 0.1%. Immerse the filter in the hot elution buffer for 15 minutes.

2. Repeat step 1 with a fresh batch of boiling elution buffer.
   Important: Do not allow the filter to dry when transferring it between batches of hot elution buffer.

3. Rinse the filter briefly in 0.01 × SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

4. Sandwich the damp filter between two sheets of Saran Wrap, and apply it to X-ray film to check that all of the probe has been removed.

5. The filter may now be dried, wrapped loosely in aluminum foil, and stored under vacuum at room temperature until needed.

REMOVING PROBES FROM NYLON MEMBRANES

1. Either
   - immerse the membrane in several hundred milliliters of 1 mM Tris · Cl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1 × Denhardt's reagent (see Table 9.1) for 2 hours at 75°C, or
   - immerse the membrane in 50% formamide, 2 × SSPE for 1 hour at 65°C.

2. Rinse the membrane briefly with 0.1 × SSPE at room temperature. Remove most of the liquid from the membrane by placing it on a pad of paper towels.

3. Sandwich the damp membrane between two sheets of Saran Wrap, and apply it to X-ray film to check that all of the probe has been removed.

4. The membrane may now be dried, wrapped loosely in aluminum foil, and stored under vacuum at room temperature until needed.

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10
Preparation of Radiolabeled DNA and RNA Probes

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Nucleic acid hybridization is a fundamental tool in molecular cloning. Virtually every aspect of cloning, characterization, and analysis of genes involves hybridization of one strand of nucleic acid to another. For example:

- The identification of specific clones in cDNA and genomic DNA libraries is frequently accomplished by colony or plaque hybridization with radiolabeled oligonucleotides or cDNA.
- The organization of specific regions of genomic DNA is often defined by Southern hybridization using segments of cloned DNA as probes.
- Analysis of gene transcription and RNA processing relies heavily on experiments in which nuclease S1 or RNAase is used to digest DNA:RNA or RNA:RNA hybrids.

The success of these techniques and others described in this manual depends on the ready availability of methods to introduce radioactive label into cloned segments of DNA or RNA copied from them. The purpose of this chapter is to describe the techniques currently used to prepare radiolabeled DNA and RNA probes.

Until the early 1970s, radioactivity could be incorporated into nucleic acids only by metabolic labeling, in which radioactive precursors (usually ^{32}P) were introduced into cells that were synthesizing the DNA of interest. This procedure required large amounts of radioactivity, involved laborious purification of the nucleic acids of interest, could be applied to only a small number of DNAs (e.g., viral genomes), and yielded probes of comparatively low specific activity ($< 1 \times 10^6$ to 2×10^6 cpm/μg). During the 1970s, two methods became available to introduce radioactivity into nucleic acids in vitro: (1) phosphorylation by bacteriophage T4 polynucleotide kinase, in which the γ-phosphate of ATP is transferred to a 5′-hydroxyl terminus in DNA or RNA (Richardson 1971); and (2) nick translation, in which *Escherichia coli* DNA polymerase I is used to replace unlabeled nucleotides in double-stranded DNA with α-^{32}P-labeled nucleotides (Kelly et al. 1970; Maniatis et al. 1975; Rigby et al. 1977). These reactions are both rapid and efficient, requiring 100- to 1000-fold less radioactive precursor than metabolic labeling while producing probes whose specific activity is an order of magnitude higher. Not surprisingly, both reactions found wide acceptance and have become a mainstay of molecular cloning.

Despite this success, radiolabeled probes prepared by these methods have certain limitations. Phosphorylation introduces a maximum of one radiolabeled atom per molecule of nucleic acid (at its 5′ terminus), thereby limiting the specific activity of the probe; nick translation, although capable of introducing radiolabeled precursors at many places along the length of a molecule, yields a fragmented probe containing the sequences of both strands of the template DNA. Consequently, in some circumstances, reassociation of the complementary strands of the probe may compete with hybridization of the radiolabeled probe to its target strand. To avoid this problem, methods were subsequently developed to synthesize single-stranded probes complementary to DNA sequences cloned in bacteriophage M13 or phagemid vectors (Ley et al. 1982; Messing 1983) (Figure 10.1). Although this ap-

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proach produces single-stranded probes of defined length and high specific activity, it requires the tedious separation of the newly synthesized probe from the unlabeled template by gel electrophoresis.

More recently, a simple and efficient method has been developed to transcribe cloned segments of DNA in vitro into highly radioactive single-stranded RNA (Melton et al. 1984) (Figure 10.2). These transcripts are now the probes of choice for most purposes, since they can be synthesized with high efficiency and can be separated from the DNA template without gel electrophoresis. Furthermore, RNA probes form hybrids with both DNA and RNA that are more stable than DNA:DNA hybrids (Casey and Davidson 1977; Meinkoth and Wahl 1984). Both DNA:RNA and RNA:RNA hybrids are resistant to digestion with RNAase, and it is therefore possible to use this enzyme to remove any probe that is nonspecifically bound without affecting the DNA:RNA or RNA:RNA hybrids.

In this chapter, we describe the methods that are commonly used to prepare radioactive DNA and RNA probes. The many uses of these probes in the isolation and analysis of segments of cloned DNA are described in other chapters.

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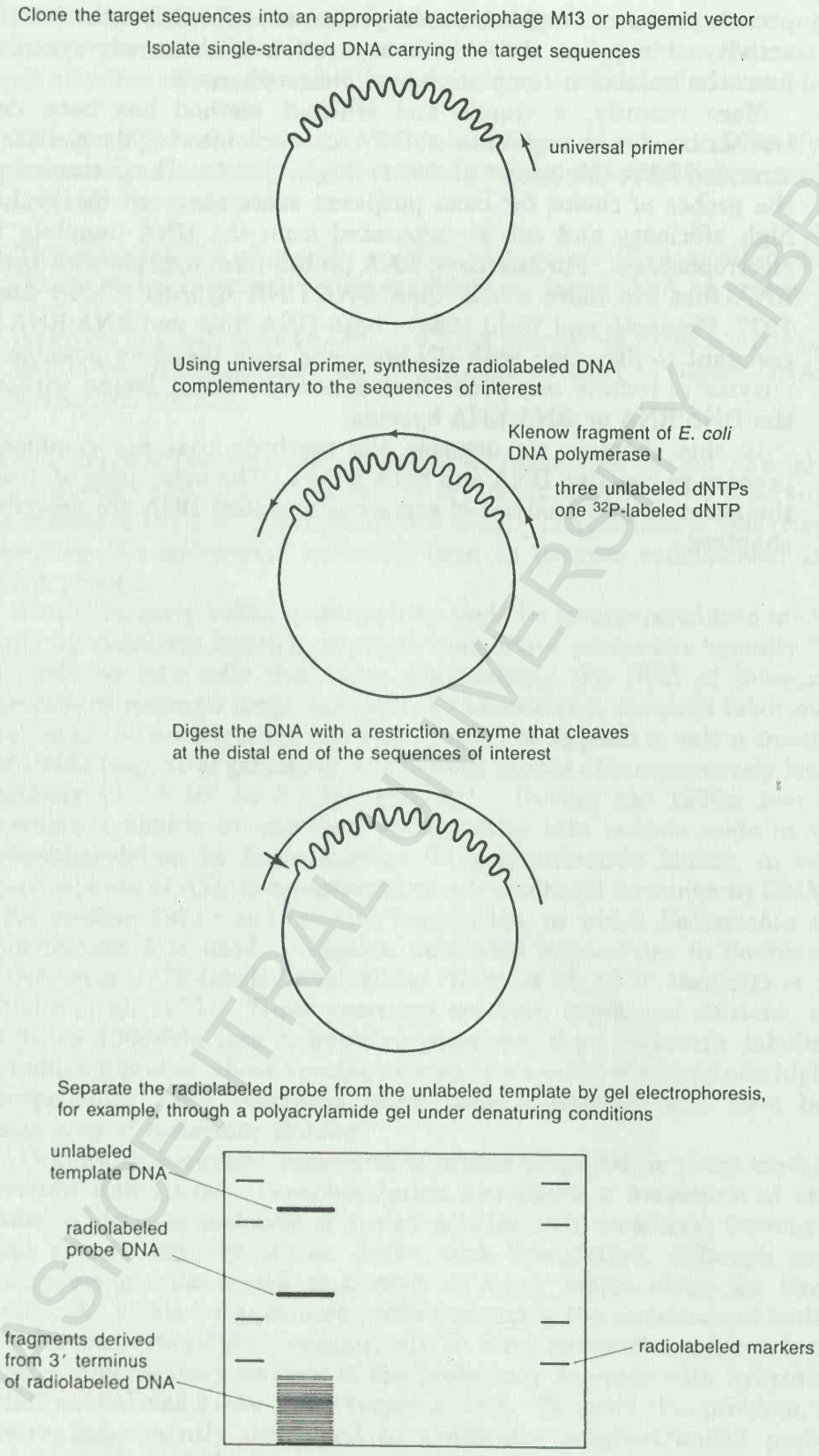
Clone the target sequences into an appropriate bacteriophage M13 or phagemid vector
Isolate single-stranded DNA carrying the target sequences
universal primer
Using universal primer, synthesize radiolabeled DNA complementary to the sequences of interest
Klenow fragment of E. coli DNA polymerase I
three unlabeled dNTPs
one 32P-labeled dNTP
Digest the DNA with a restriction enzyme that cleaves at the distal end of the sequences of interest
Separate the radiolabeled probe from the unlabeled template by gel electrophoresis, for example, through a polyacrylamide gel under denaturing conditions
unlabeled template DNA
radiolabeled probe DNA
fragments derived from 3′ terminus of radiolabeled DNA
radiolabeled markers

FIGURE 10.1
Synthesis of probes from single-stranded DNA vectors.

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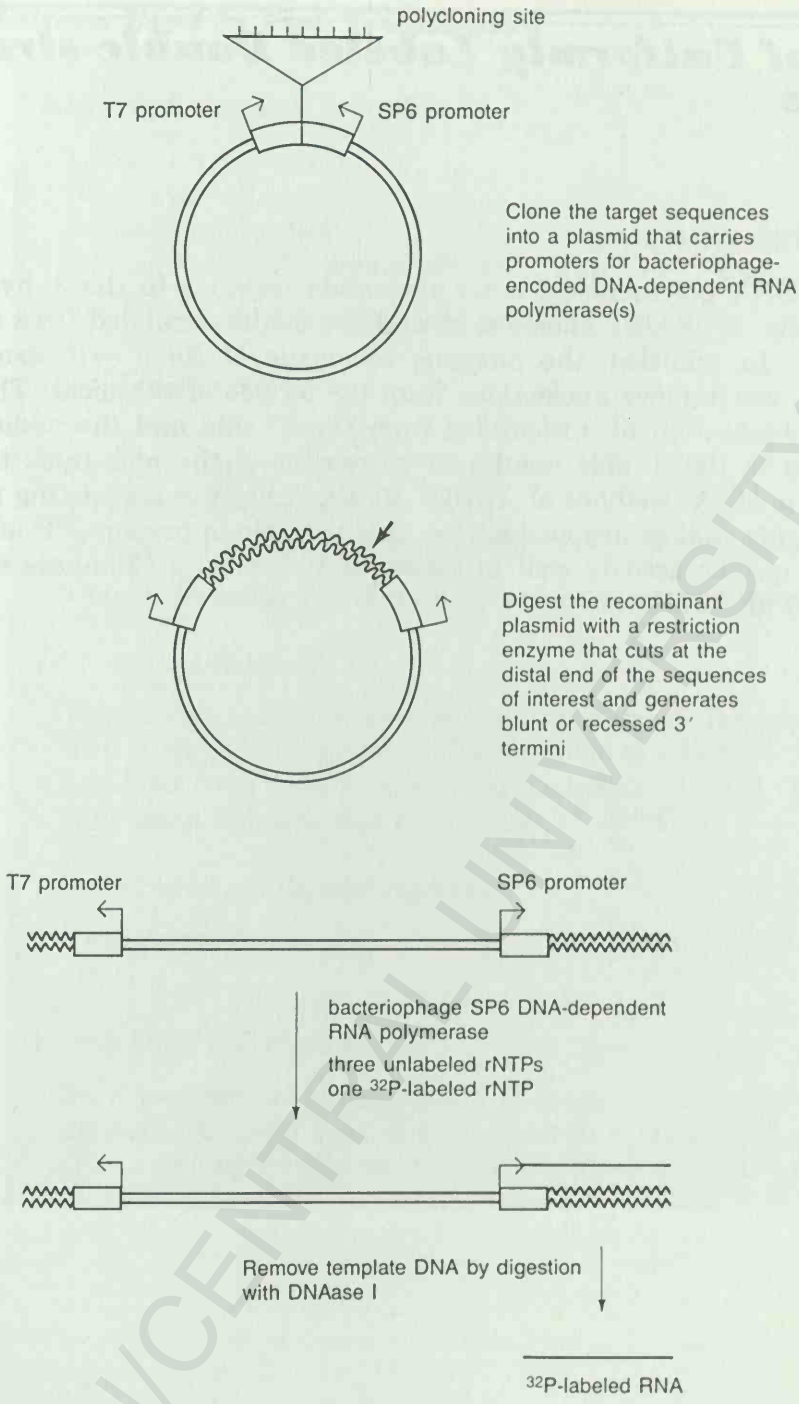
polycloning site
T7 promoter
SP6 promoter
Clone the target sequences into a plasmid that carries promoters for bacteriophage-encoded DNA-dependent RNA polymerase(s)
Digest the recombinant plasmid with a restriction enzyme that cuts at the distal end of the sequences of interest and generates blunt or recessed 3′ termini
T7 promoter
SP6 promoter
bacteriophage SP6 DNA-dependent RNA polymerase
three unlabeled rNTPs
one 32P-labeled rNTP
Remove template DNA by digestion with DNAase I
32P-labeled RNA

FIGURE 10.2
Synthesis of RNA probes by in vitro transcription.

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Synthesis of Uniformly Labeled Double-stranded DNA Probes

NICK TRANSLATION OF DNA

E. coli DNA polymerase I adds nucleotide residues to the 3′-hydroxyl terminus that is created when one strand of a double-stranded DNA molecule is nicked. In addition, the enzyme, by virtue of its 5′→3′ exonucleolytic activity, can remove nucleotides from the 5′ side of the nick. The simultaneous elimination of nucleotides from the 5′ side and the addition of nucleotides to the 3′ side results in movement of the nick (nick translation) along the DNA (Kelly et al. 1970). By replacing the preexisting nucleotides with highly radioactive nucleotides, it is possible to prepare ^{32}P-labeled DNA with a specific activity well in excess of 10^8 cpm/μg (Maniatis et al. 1975; Rigby et al. 1977).

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Stock Solutions Used in Nick Translation

10 × Nick-translation buffer

0.5 M Tris · Cl (pH 7.5)
0.1 M $MgSO_4$
1 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma)

Divide the stock solution into small aliquots and store them at −20°C.

10 × Ligation buffer

0.5 M Tris · Cl (pH 7.6)
100 mM $MgCl_2$
100 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

Divide the stock solution into small aliquots and store them at −20°C.

Pancreatic DNAase I

Prepare a stock solution containing pancreatic DNAase I (1 mg/ml) in 0.15 M NaCl, 50% glycerol. (Electrophoretically pure DNAase I can be obtained from a number of manufacturers.) Divide the stock solution into small aliquots and store them at −20°C.

Deoxyribonucleoside triphosphates

Prepare stock solutions of each dNTP (20 mM) as described in Appendix C.

E. coli *DNA polymerase I*

Most commercial suppliers sell this enzyme in buffer containing 50% glycerol. Usually 1 μl of the preparation contains 5 units of enzyme. (For a definition of a unit, see Richardson et al. 1964.)

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Protocol for Nick Translation

1. Mix:

10 × nick-translation buffer	2.5 μl
DNA	0.5 μg
unlabeled dNTPs (if needed)	20 nmoles of each
[α-^{32}P]dNTP	16 pmoles
H_2O to 21.5 μl	

Chill the mixture to 0°C.

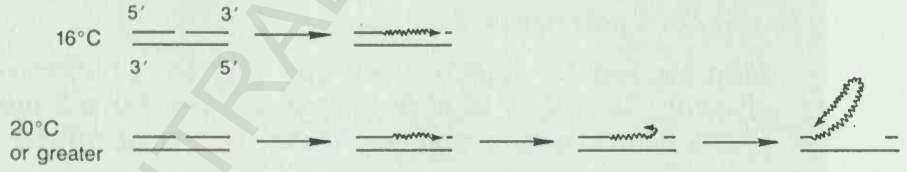
For unlabeled dNTPs, add 1 μl of a 20 mM solution of each unlabeled dNTP; usually, 20 nmoles of three unlabeled dNTPs are added to the nick-translation mixture, e.g., dTTP, dATP, and dGTP. Using this example, 5 μl of aqueous [α-^{32}P]dCTP (sp. act. >3000 Ci/mmole; 10 μCi/μl) would then be added.

2. Make a 10^5-fold dilution of a small quantity of a stock solution of pancreatic DNAase I (1 mg/ml) in ice-cold 1 × nick-translation buffer containing 50% glycerol. The diluted enzyme is stable when stored at −20°C in this buffer.

3. Add 2.5 μl of the diluted DNAase I (10 ng/ml) to the reaction. Mix by vortexing.

4. Add 2.5 units of *E. coli* DNA polymerase I. Mix by gentle vortexing.

5. Incubate the reaction for 60 minutes at 16°C. If the reaction is carried out at higher temperature, a considerable amount of "snapback" DNA may be generated by *E. coli* DNA polymerase I copying the newly synthesized strand.



A typical reaction contains 0.5 μg of DNA in a volume of 25 μl. However, the reaction works well with as little as 20 ng of DNA and can be scaled down to volumes as small as 5 μl.

Although *E. coli* DNA polymerase I will work with concentrations of dNTPs as low as 2 μM, the enzyme synthesizes DNA much more efficiently when supplied with higher concentrations of substrates. For reasons of cost, nick-translation reactions usually contain minimal concentrations of radiolabeled dNTPs (0.5–5 μM) and much greater concentrations of unlabeled dNTPs (1 mM). A 25-μl reaction therefore contains 20 nmoles of each unlabeled dNTP and 5–16 pmoles of each radiolabeled dNTP. The specific activity of the final product depends in large part on the specific activity of the radiolabeled dNTP used in the reaction. By diluting the radiolabeled dNTP with the homologous unlabeled dNTP, it is therefore possible to prepare DNA labeled to different specific activities. If high specific activities (>10^8 cpm/μg) are required (e.g., for screening of recombinant DNA libraries or for detecting single-copy sequences in Southern hybridizations of eukaryotic DNA), the nick-translation reaction should contain all four radiolabeled dNTPs (sp. act. >800 Ci/mmole) and no unlabeled dNTPs. For most other purposes, it is adequate to use one dNTP labeled with

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α-^{32}P and three unlabeled dNTPs or to dilute each [α-^{32}P]dNTP with an appropriate amount of the unlabeled dNTP.

Most commercial suppliers sell [α-^{32}P]dNTPs in a concentrated aqueous solution, which can be added directly to the nick-translation reaction. The specific activities range from 400 to >3000 Ci/mmole. If [α-^{32}P]dNTPs in an ethanol/water mixture are used, remove the ethanol and water as described in Appendix E.

6. Stop the reaction by adding 1 μl of 0.5 M EDTA (pH 8.0).

7. Using the DE-81 binding or trichloroacetic acid (TCA) precipitation assay described in Appendix E, determine the proportion of [α-^{32}P]dNTPs incorporated into the DNA.

8. Separate the radiolabeled DNA from the unincorporated dNTPs by

   - chromatography on, or centrifugation through, a small column of Sephadex G-50 (see Appendix E) or
   - selective precipitation of nick-translated DNA by ethanol (see Appendix E).

Notes

i. The specific activity of the nick-translated DNA depends not only on the specific activity of the dNTPs, but also on the extent of nucleotide replacement of the template. This can be controlled by varying the amount of DNAase I in the reaction. The aim is to establish conditions that will result in incorporation of about 30% of the [α-^{32}P]dNTPs into DNA.

ii. The amount of DNAase I added to the reaction and the amount of DNAase contaminating the preparation of *E. coli* DNA polymerase I also affect the size of the DNA after nick translation. Because the amount of contaminating nuclease differs widely between different batches of commercial *E. coli* DNA polymerase I, each new preparation should be tested as follows:

   a. Set up five nick-translation reactions. Add *E. coli* DNA polymerase I to all of the tubes and different quantities of DNAase I as shown in Table 10.1.

TABLE 10.1

	DNAase I (μl)	
Tube	**0.01 μg/ml**	**0.1 μg/ml**
1	0	0
2	1	0
3	2	0
4	4	0
5	0	1

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b. Use the DE-81 binding or TCA precipitation assay (see Appendix E) to determine the proportion of the [α-^{32}P]dNTPs incorporated into the DNA.

c. Analyze the size of the DNA by electrophoresis through an alkaline agarose gel (see Chapter 6, pages 6.20–6.21), using as markers end-labeled fragments of DNA of known size.

d. Choose a concentration of DNAase I that results in incorporation of about 30% of the [α-^{32}P]dNTPs into the DNA. The labeled DNA strands should be 400–800 nucleotides in length.

iii. Nick translation usually yields uniformly labeled DNA (Rigby et al. 1977), indicating that any bias in nicking by DNAase I (Ehrlich et al. 1973) or in synthesis by *E. coli* DNA polymerase I is too small to produce significant distortion in the pattern of labeling.

iv. The enzymes used in nick translation are sensitive to contaminants in agarose. Thus, DNA samples eluted from agarose gels should be carefully purified as described in Chapter 6, pages 6.32–6.35, before they are used in nick-translation reactions (see also alternative method for nick translation, pages 10.11–10.12).

v. For some purposes (e.g., analysis of DNA:RNA hybrids by nuclease S1), it is desirable to obtain a uniformly labeled probe whose chain length is significantly longer than 400–800 nucleotides. Three options are available in this case: A DNA probe of defined length can be synthesized by primer extension on a single-stranded DNA template (see pages 10.22–10.24), an RNA probe of defined length can be synthesized in vitro using a bacteriophage DNA-dependent RNA polymerase, or the products of the nick-translation reaction can be treated with ligase as follows:

a. Carry out the nick-translation reaction (steps 1–5) using the amount of DNAase I determined to be optimal as described above.

b. Add 1 μl of 0.5 M EDTA (pH 8.0). Heat the reaction for 5 minutes at 70°C to inactivate *E. coli* DNA polymerase I and DNAase I.

c. Add 150 μl of 10 mM $MgCl_2$, 20 μl of 10 mM ATP, and 20 μl of 10 $\times$ ligation buffer (see page 10.7).

d. Add 1–2 units of bacteriophage T4 DNA ligase, and incubate the reaction for 2 hours at room temperature.

e. Separate the nick-translated and ligated DNA from unincorporated dNTPs either by chromatography on a column of Sephadex G-50 or by spun-column chromatography (see Appendix E).

f. Analyze the size of the DNA by electrophoresis through an alkaline agarose gel (see Chapter 6, pages 6.20–6.21).

g. If necessary, isolate DNA of the required size from a preparative alkaline agarose gel.

Alternative Protocol for Nick Translation

The efficiency of nick translation can vary considerably depending on the DNA template. For example, small-scale preparations of plasmid and bacteriophage DNA often contain contaminants that inhibit the incorporation of labeled nucleotides. When the DNAase I and *E. coli* DNA polymerase I reactions are carried out simultaneously, a lag is observed before incorporation of radiolabeled nucleotides begins (Rigby et al. 1977). The lag is thought to reflect the time required for the DNAase I to generate nicks in the template. Interestingly, the lag is longer when using preparations of DNA that give lower efficiencies of label incorporation. It therefore seems that DNAase I is rate-limiting in the reaction and that the enzyme may be sensitive to contaminants found in preparations of DNA. To minimize this problem, the DNAase I and *E. coli* DNA polymerase I reactions can be carried out sequentially, rather than simultaneously (Koch et al. 1986). In the protocol described below, the DNA template is first digested with DNAase I at 37°C, the reaction is then diluted, and synthesis with *E. coli* DNA polymerase I is carried out at 16°C (to inhibit the formation of snapback DNA). The activity of the DNAase I is reduced 20-fold by dilution and approximately 8-fold by lowering of the temperature, so there is little or no degradation of DNA during the polymerization reaction. It is therefore possible to allow synthesis to proceed for extended periods of time, resulting in the incorporation of up to 90% of the radiolabeled dNTP and the synthesis of probes with specific activities in excess of 10^9 cpm/μg (Koch et al. 1986).

1. In a microfuge tube, mix 0.5 μg of double-stranded DNA (in a volume of 1 μl) with 1 μl of 3 × nick-translation buffer and 0.5 ng of DNAase I (in a volume of 1 μl). Incubate for 15 minutes at 37°C.

   Each new batch of DNAase I should be titrated to give DNA that is approximately 300–400 nucleotides in length after digestion, as assayed by electrophoresis through an alkaline agarose gel (see Chapter 6, pages 6.20–6.21).

2. Place the reaction on ice and add:

10 × nick-translation buffer (see page 10.7)	1 μl
unlabeled dNTPs (a solution containing three of the four dNTPs, each at a concentration of 5 mM)	1 μl
[α-^{32}P]dNTP (sp. act. >3000 Ci/mmole; 10 μCi/μl in aqueous solution)	5 μl

   Initiate synthesis by adding 1 μl of *E. coli* DNA polymerase I (~5 units). Mix the reagents by gently tapping the outside of the tube. Centrifuge the tube briefly (1–2 seconds) to get all the liquid to the bottom.

   Incorporation of radioactivity into DNA is linear for several hours. To obtain probes of very high specific activity, incubate the reaction at 16°C for several hours until virtually all of the radiolabeled precursor has been incorporated into the DNA. To monitor the course of the reaction, measure the proportion of radiolabel that becomes incorporated into trichloroacetic acid (TCA)-precipitable material (see Appendix E).

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3. Add 20 μl of Buffer A, and store the probe at −20°C until it is needed.

Buffer A

50 mM Tris · Cl (pH 7.5)
50 mM NaCl
5 mM EDTA (pH 8.0)
0.5% SDS

Notes

i. Probes radiolabeled to high specific activity are rapidly damaged by radiochemical decay and should be used without delay.

ii. Since the bulk of the radioactive precursor is incorporated into the DNA, it is not usually necessary to purify the probe before hybridization. If need be, the radiolabeled probe can be separated from the unincorporated dNTPs by

- chromatography on, or centrifugation through, a small column of Sephadex G-50 (see Appendix E) or
- selective precipitation of nick-translated DNA by ethanol (see Appendix E).

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SYNTHESIS OF UNIFORMLY LABELED DNA PROBES USING RANDOM OLIGONUCLEOTIDE PRIMERS

It has been known for many years that oligonucleotides can serve as primers for initiation of DNA synthesis on single-stranded templates by DNA polymerases (see, e.g., Goulian 1969). If the oligonucleotides are heterogeneous in sequence, they will form hybrids at many positions, so that every nucleotide of the template (except, perhaps, those at the extreme 5′ terminus) will be copied at equal frequency into the product. By using [α-^{32}P]dNTPs as precursors, DNA probes radiolabeled to very high specific activity can be synthesized in this way.

Random primers can be obtained in three ways:

- By digesting calf thymus or salmon sperm DNA with DNAase I to generate a large population of single-stranded DNA fragments 6–12 nucleotides in length (see Feinberg and Vogelstein 1983).
- By purchasing random oligonucleotides prepared from calf thymus DNA from commercial sources (e.g., Pharmacia, Boehringer Mannheim, etc.).
- By synthesizing on an automated DNA synthesizer a population of octamers that contains all four bases in every position. Such completely redundant populations of synthetic oligomers can also be purchased from International Biotechnologies. Because of their uniform length and lack of sequence bias, synthetic oligonucleotides are the primers of choice for synthesis of radioactive copies of single-stranded templates.

The type of DNA polymerase used depends on the nature of the template: RNA-dependent DNA polymerase (reverse transcriptase) is used to copy single-stranded RNA templates, whereas the Klenow fragment of *E. coli* DNA polymerase I is used when the template is single-stranded DNA. In both cases, synthesis of DNA is carried out using one [α-^{32}P]dNTP and three unlabeled dNTPs as precursors to yield probes whose specific activities are 5×10^8 to 4×10^9 cpm/μg. These high specific activities are achieved because a large proportion of the radiolabeled precursor is incorporated into the product.

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SYNTHESIS OF RADIOLABELED PROBES BY PRIMER EXTENSION

This method is used to generate probes from denatured, closed circular DNA or denatured, linear, double-stranded DNA (e.g., restriction fragments purified by gel electrophoresis; Feinberg and Vogelstein 1983, 1984). The purified DNA, mixed with a molar excess of primers, is denatured by boiling, and synthesis is then carried out using the Klenow fragment of *E. coli* DNA polymerase I. This enzyme lacks $5' \rightarrow 3'$ exonuclease activity, so that the radioactive product is synthesized exclusively by primer extension rather than by nick translation and is not degraded exonucleolytically. The reaction is carried out at pH 6.6, where the $3' \rightarrow 5'$ exonuclease activity of the enzyme is much reduced (Lehman and Richardson 1964). These conditions favor random initiation of synthesis, since oligonucleotide primers bound to the template are not degraded enzymatically.

1. On a small piece of Parafilm, mix 200 ng of double-stranded DNA (in a volume of 1 μl) with 75 ng of random primers (also in a volume of 1 μl). Draw the mixture into the center of a disposable 10-μl glass micropipette. Seal the ends of the pipette in the flame of a Bunsen burner. Immerse the sealed pipette in boiling water for 3 minutes.

   The specific activity of the product depends on the amount of template added to the reaction: the lower the amount of template, the greater the specific activity. Under the reaction conditions described, radiolabeling to maximum specific activity is achieved with approximately 200 ng of template. However, probes labeled to high specific activity degrade rapidly due to radiochemical decay (Stent and Fuerst 1960). Probes whose specific activities exceed 2×10^9 cpm/μg must therefore be used without delay. Probes radiolabeled to lower specific activity (10^8 cpm/μg) are more than adequate for most purposes and can be stored for several days.

   The average size of the probe is an inverse function of the concentration of primer (Hodgson and Fisk 1987). The presence of high concentrations of primer is thought to lead to steric hindrance or to exhaustion of a precursor. Under the reaction conditions described, the length of the radiolabeled product is approximately 400–600 bp, as determined by electrophoresis through an alkaline agarose gel (see Chapter 6, pages 6.20–6.21).

2. Meanwhile, in a small microfuge tube (0.5 ml) stored in an ice bath, mix:

20 mM dithiothreitol	1 μl
a solution of dGTP, dCTP, and dTTP, each at a concentration of 5 mM	1 μl
10 × RP buffer	1 μl
[α-^{32}P]dATP (sp. act. > 3000 Ci/mmole; 10 μCi/μl)	3 μl
H_2O	1 μl

10 × RP buffer

900 mM HEPES (adjusted to pH 6.6 with 4 N NaOH)
100 mM $MgCl_2$

3. Withdraw the sealed glass pipette from the boiling water and quickly immerse it in a tray of ice water. After 1 minute, remove the sealed pipette and dry the outside with Kimwipes. Open both ends of the pipette with a glass file, and transfer its contents to the small microfuge tube.

4. Add 5 units (~1 μl) of the Klenow fragment of *E. coli* DNA polymerase I. Mix the components by gently tapping the outside of the tube. Centrifuge at 12,000*g* for 1–2 seconds in a microfuge to move all the liquid to the bottom. Incubate the reaction for at least 3 hours at room temperature.

   The rate of incorporation depends on the concentrations of the template and the radioactive precursor. In the standard reaction, approximately 90% of the [α-^{32}P]dNTP, which is the rate-limiting component, is incorporated over a period of 2–3 hours. However, if the concentration of template is reduced or the concentration of [α-^{32}P]dATP is increased, it will take longer to achieve maximal incorporation of radiolabel. Because the reaction is carried out under conditions that minimize exonucleolytic digestion, the radiolabeled product is not degraded to a detectable extent. If necessary, therefore, the reaction can be allowed to proceed for 12–16 hours without harm.

   To monitor the course of the reaction, measure the proportion of radiolabel that becomes incorporated into trichloroacetic acid (TCA)-precipitable material (see Appendix E).

5. Add 10 μl of Buffer A to the reaction, and store the radiolabeled probe at −20°C until it is needed.

Buffer A

50 mM Tris · Cl (pH 7.5)
50 mM NaCl
5 mM EDTA (pH 8.0)
0.5% SDS

Notes

i. Probes radiolabeled to high specific activity are rapidly damaged by radiochemical decay and should be used without delay.

ii. Since the bulk of the radioactive precursor is incorporated into DNA, it is not usually necessary to purify the probe before hybridization. If need be, the radiolabeled probe can be separated from the unincorporated dNTPs by

   - chromatography on, or centrifugation through, a small column of Sephadex G-50 (see Appendix E) or
   - selective precipitation of nick-translated DNA by ethanol (see Appendix E).

iii. Radiolabeled probes prepared by random priming are usually 400–600 nucleotides in length as determined by electrophoresis through alkaline agarose gels.

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RADIOLABELING OF DNA IN THE PRESENCE OF MELTED AGAROSE

A variation of the method described above can be used to radiolabel DNA in slices cut from gels cast with low-melting-temperature agarose in Tris-acetate electrophoresis buffer (see Chapter 6) (Feinberg and Vogelstein 1984).

1. After electrophoresis (in Tris-acetate electrophoresis buffer), stain the gel with ethidium bromide (0.5 μg/ml in water), and excise the desired band.

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

2. Place the band in a preweighed microfuge tube and measure its weight. Add 3 ml of H_2O for every gram of agarose gel.

3. Place the microfuge tube in a boiling-water bath for 7 minutes to melt the gel and denature the DNA. If radiolabeling is to be carried out immediately, store the tube at 37°C until the template is required. Otherwise, store the tube at −20°C. After each removal from storage, the DNA/gel slurry should be heated to 100°C for 3–5 minutes and then stored at 37°C until the radiolabeling reaction is initiated.

4. To a fresh microfuge tube, add in the following order:

5 × oligonucleotide-labeling buffer	10 μl
bovine serum albumin (Fraction V; Sigma) (10 mg/ml)	2 μl
DNA in a volume no greater than 32 μl	20–50 ng
[α-^{32}P]dCTP (sp. act. > 3000 Ci/mmole; 10 μCi/μl)	5 μl
Klenow fragment of *E. coli* DNA polymerase I (5 units)	1 μl
H_2O to 50 μl	

   Incubate the reaction at room temperature for 3–12 hours (depending on the amount of template).

5 × Oligonucleotide-labeling buffer

250 mM Tris · Cl (pH 8.0)
25 mM $MgCl_2$
5 mM β-mercaptoethanol
2 mM each dATP, dGTP, dTTP
1 M HEPES (adjusted to pH 6.6 with 4 N NaOH)
1 mg/ml oligonucleotides

This buffer should be stored in small aliquots at −20°C. It may be frozen and thawed several times without harm.

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5. Add 200 μl of Buffer A to the reaction, and store the radiolabeled probe at −20°C until it is needed.

Buffer A

50 mM Tris · Cl (pH 7.5)
50 mM NaCl
5 mM EDTA (pH 8.0)
0.5% SDS

Notes

i. Probes radiolabeled to high specific activity are rapidly damaged by radiochemical decay and should be used without delay.

ii. Since the bulk of the radioactive precursor is incorporated into DNA, it is not usually necessary to purify the probe before hybridization. If need be, the radiolabeled probe can be separated from the unincorporated dNTPs by

- chromatography on, or centrifugation through, a small column of Sephadex G-50 (see Appendix E) or
- selective precipitation of nick-translated DNA by ethanol (see Appendix E).

iii. Radiolabeled probes prepared by random priming are usually 400–600 nucleotides in length as determined by electrophoresis through alkaline agarose gels.

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Preparation of Single-stranded Probes

Single-stranded probes, which consist of only one of the two complementary strands of a given sequence of nucleic acid, have a considerable advantage over the more traditional double-stranded probes: The absence of a complementary strand eliminates the possibility of forming nonproductive hybrids composed solely of reannealed probe. This is especially valuable when a probe derived from one species is used to detect cross-hybridizing sequences in a distantly related species. If the degree of mismatch is high, hybrids formed between the complementary sequences of double-stranded probes are more stable than those formed between the probe and the target sequences. The resulting segregation of the probe leads to a decrease in the sensitivity with which the target sequences can be detected.

The absence of a competing complementary strand is particularly beneficial in experiments involving hybridization between RNA and DNA. For example, when using single-stranded DNA probes in nuclease-S1 mapping experiments (see Chapter 7), there is no need to use special hybridization conditions to suppress the formation of unwanted DNA:DNA hybrids. Finally, the availability of single-stranded probes of known polarity greatly simplifies the task of determining which strand of a particular segment of DNA is transcribed into mRNA.

Single-stranded probes are prepared in vitro from DNA templates by (1) synthesis of radiolabeled DNA that is complementary to sequences cloned in a phagemid or bacteriophage M13 vector or (2) transcription into radiolabeled RNA of target sequences cloned in a vector carrying a promoter for a bacteriophage DNA-dependent RNA polymerase. Both methods yield probes of extremely high specific activity and defined length. However, in the first method, it is necessary to use physical methods to separate the newly synthesized radiolabeled DNA from the complementary unlabeled template. In the second method, the DNA template is more easily removed by digestion with RNAase-free DNAase I. For this reason, in vitro transcription of cloned DNA fragments is rapidly becoming the method of choice to generate single-stranded probes.

Single-stranded probes can also be prepared from RNA templates using either oligo(dT) or oligonucleotides of random sequence to prime the synthesis of cDNA. Probes of this type may be used directly to screen genomic DNA or cDNA libraries; more commonly, however, the concentration of the sequences of interest is increased by subtractive hybridization before the probe is used for screening.

In this section, we describe the following techniques:

- Synthesis and purification of single-stranded DNA probes
- In vitro transcription of double-stranded DNA templates into radiolabeled RNA
- Preparation of cDNA probes that may be used for differential and subtractive screening of genomic DNA and cDNA libraries

PRODUCTION OF SINGLE-STRANDED DNA PROBES USING BACTERIOPHAGE M13 VECTORS

In this method, a synthetic oligonucleotide is annealed to single-stranded DNA derived from a recombinant bacteriophage M13 or phagemid and is used to prime the synthesis of complementary radiolabeled DNA (see Figure 10.1, page 10.4). Most commonly, the primer is complementary to a region of the *lac* gene immediately 3′ to the polycloning site in the mp series of bacteriophage M13 vectors (Yanisch-Perron et al. 1985). This "universal" primer, which is sold by several companies, has the advantage that it can be used to prepare probes complementary to any segment of DNA that has been cloned into any restriction site in the polycloning site of the vector. However, custom-made oligonucleotides complementary to specific sequences within the cloned DNA can also be used to prepare probes that represent only a portion of the cloned sequences.

Synthesis of the probe is carried out with the Klenow fragment of *E. coli* DNA polymerase I, using as substrates one [α-^{32}P]dNTP and three unlabeled dNTPs. Under standard conditions, the rate of the reaction (and therefore the size of the product) is limited by the concentration of the radiolabeled dNTP in the reaction. The effects of varying the amounts of radiolabeled and unlabeled dNTPs on the size and specific activity of the probe are discussed below. The products of the reaction are partially double-stranded molecules that contain varying amounts of radioactivity (see Figure 10.1). These heterogeneous products are digested with a restriction enzyme that cleaves either within or downstream from the cloned sequences to produce a partially double-stranded DNA that consists of a radioactive probe hydrogen-bonded to the unlabeled single-stranded template. The probe is then separated from the template and from smaller fragments of DNA by gel electrophoresis (e.g., through a polyacrylamide gel under denaturing conditions [see Chapter 11]) or, in the case of very small probes, by alkaline chromatography through Sepharose CL-4B (see page 10.25). The specific activity of probes generated in this way can exceed 10^9 cpm/μg.

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Primer:Template Ratios and Nucleotide Concentrations

The size, specific activity, and yield of the radioactive strand synthesized by the Klenow fragment of *E. coli* DNA polymerase I are affected by the relative concentrations of primer, template, and dNTPs. The K_m of the enzyme is different for each of the four dNTPs (Travaglini et al. 1975):

dGTP	45 μM
dCTP	200 μM
dATP	12 μM
dTTP	140 μM

When the concentration of each of the four dNTPs in the reaction is greater than K_m, the synthesis of DNA occurs at a maximal rate. If the concentration of any one of the four precursors falls below K_m, the rate of synthesis drops dramatically. Thus, when each of the four dNTPs is present at a concentration less than K_m, as is often the case when only radiolabeled nucleotides are used as precursors, the yield of product can be extremely low. To avoid this problem, primer-extension reactions are usually carried out with three unlabeled dNTPs each at a concentration in excess of K_m and one radiolabeled dNTP at a lower concentration, which is usually below K_m. Under these conditions, the proportion of radiolabel that is incorporated into DNA is very high, even though the rate of the reaction may be far from maximal.

When one or more precursors are present at limiting concentrations, the size of the product may be unacceptably small unless the amount of template in the reaction is also reduced. For example, in a reaction containing three unlabeled dNTPs each at a concentration in excess of K_m, 16 pmoles (50 μCi) of a radiolabeled dNTP (sp. act. 3000 Ci/mmole), and 2 pmoles of template ($\sim$1 μg), an average of only 28 nucleotides will be added to each primer molecule before the synthesis is brought to a halt by a lack of the radiolabeled dNTP. However, if the template concentration is reduced to 0.02 pmoles ($\sim$10 ng), an average of 3000 nucleotides will be added to each primer before the pool of precursors is exhausted.

To maximize the efficiency with which template molecules are used, the primer must be present in molar excess and the concentrations of primer and template must be high enough to drive the formation of primer:template hybrids to completion. These conditions are met when the primer is present in tenfold molar excess and its concentration is 200 pmoles/ml or greater. For some purposes, however, it is desirable to utilize the primer efficiently (e.g., when the primer is end-labeled). In this case, the primer:template ratio is reversed, so that the reaction contains a five- to tenfold molar excess of template.

Primer-extension reactions are almost always carried out with an excess of enzyme. If the enzyme is limiting, the number of DNA molecules initiated in the reaction (and hence the overall yield) will be reduced. However, the newly synthesized DNA molecules will be longer, on average, than DNA molecules synthesized when the enzyme is in excess.

Because it is possible to affect the size, specific activity, and yield of the probe by adjusting the conditions of the reaction, it is important to decide

what particular combination of properties would be best-suited for the task at hand. In northern and Southern hybridizations, when maximal sensitivity is often the overriding concern, the amount of template in the reaction should be kept to a minimum and the concentration of radiolabeled dNTP(s) should be increased as much as is practicable. On the other hand, when uniformly labeled probes are prepared for analysis of mRNAs by nuclease S1, the yield of full-length probe may be more important than its specific activity. In this case, the radiolabeled dNTP may be supplemented with unlabeled precursor until their combined concentration approaches K_m. The incorporation of radioactivity into trichloroacetic acid (TCA)-precipitable material (see Appendix E) is used to measure the yield of the reaction, and the size of the product is assayed by electrophoresis through an alkaline agarose gel (see Chapter 6).

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Synthesis of Single-stranded DNA Probes

This method was first used by Ley et al. (1982).

1. Mix:

single-stranded template (bacteriophage M13 or phagemid DNA) (~0.5 pmoles)	1 μg
the appropriate primer	5 pmoles
10× Klenow salt solution	3 μl
H_2O to 20 μl	

10× Klenow salt solution

0.5 M NaCl
0.1 M Tris·Cl (pH 7.5)
0.1 M $MgCl_2$

2. Heat the mixture to 85°C for 5 minutes and then let it cool slowly to 37°C. This can be achieved by floating the tube in a piece of Styrofoam in a 250-ml beaker filled with water equilibrated to 85°C. Store the beaker at room temperature until the temperature of the water falls to 37°C (~30 minutes).

3. Add:

0.1 M dithiothreitol	2 μl
[α-^{32}P]dATP (sp. act. 3000 Ci/mmole; 10 μCi/μl; 16 pmoles)	5 μl
a 40 μM solution of unlabeled dATP	1 μl
a solution containing dCTP, dTTP, and dGTP, each at a concentration of 20 mM	1 μl

Mix well by vortexing. Centrifuge the tube at 12,000*g* for 1–2 seconds in a microfuge to move all of the liquid to the bottom.

4. Transfer 0.5 μl of the mixture to a microfuge tube containing 15 μl of 20 mM EDTA (pH 8.0). Store the tube on ice.

5. Add 1 μl (5 units) of the Klenow fragment of *E. coli* DNA polymerase I to the remainder of the mixture. Mix the components of the reaction by tapping the side of the tube. Incubate the reaction for 30 minutes at room temperature.

6. Transfer 0.5 μl of the reaction to a fresh microfuge tube containing 20 μl of 0.5 M EDTA (pH 8.0). Store the tube on ice.

7. Add 1 μl of a 20 mM solution of unlabeled dATP to the remainder of the reaction. Mix by tapping the side of the tube. Incubate the reaction for a further 20 minutes at room temperature.

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Meanwhile, using the samples stored in steps 4 and 6, determine the fraction of radioactivity that has become insoluble in 10% trichloroacetic acid (TCA) as described in Appendix E.

The addition of unlabeled dATP increases the concentration of the precursor approximately 500-fold and ensures that all newly synthesized products will be extended past the site of restriction enzyme cleavage.

If the reaction has gone well, 50–80% of the radioactivity will have been incorporated into DNA. Even though the proportion of the radiolabel that has been incorporated is large, the length of the newly synthesized product is small when the sample is withdrawn from the reaction (step 6). For example, suppose that 50% of the [α-^{32}P]dATP is incorporated into DNA. Since the reaction contained a total of 56 pmoles of dATP (16 pmoles radiolabeled and 40 pmoles unlabeled), a total of 28 pmoles of dATP has been incorporated into DNA. Assuming that each of the four dNTPs is equally represented in the product, a total of $4 \times 28 = 112$ pmoles of dNTP has been incorporated into DNA. If primer binds to every molecule of template DNA in the reaction and if the Klenow fragment of *E. coli* DNA polymerase I is in excess, then the average length of the product will be 224 nucleotides.

8. Heat the reaction to 68°C for 10 minutes to inactivate the Klenow fragment of *E. coli* DNA polymerase I. Adjust the concentration of NaCl in the reaction to achieve optimal conditions for cleavage of the product by the selected restriction enzyme. Remember that the concentration of NaCl in the primer-extension reaction is 50 mM.

   If the restriction enzyme works best in the absence of NaCl, the products of the primer-extension reaction should be purified by extraction with phenol:chloroform and precipitation with ethanol. Then redissolve the DNA in 27 μl of TE (pH 7.6) and add 3 μl of the appropriate 10× restriction buffer.

9. Add 20 units of the desired restriction enzyme, and incubate the reaction at the appropriate temperature for 1 hour.

10. Purify the DNA by extraction with phenol:chloroform, and remove unincorporated dNTPs by spun-column chromatography or differential precipitation with ethanol (see Appendix E for these procedures). Add 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM.

    It is essential that no free magnesium ions are present when the DNA is exposed to alkali (step 11). Otherwise, the DNA will form an insoluble complex with $Mg(OH)_2$.

11. Isolate the radiolabeled DNA by electrophoresis through a denaturing polyacrylamide or alkaline agarose gel (see Chapter 11, pages 11.22–11.27, or Chapter 6, pages 6.20–6.21, respectively), depending on the size of the fragment.

12. After electrophoresis, cover the gel with Saran Wrap. Attach a set of adhesive dot labels marked with radioactive ink around the edge of the sample on the Saran Wrap. Cover the dots with Scotch Tape. This prevents contamination of the filmholder or intensifying screen with the radioactive ink. Expose the gel to autoradiographic film for 5–10 minutes.

    Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot

(> 2000 cps on a hand-held minimonitor), hot (500–2000 cps on a hand-held minimonitor), and cool (50–500 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

13. After developing the film, use the radioactive dots to align it with the gel. Cut a hole in the film over the position of the desired fragment of DNA. Use the hole as a template to mark the position of the DNA on the Saran Wrap covering the gel. Remove the film and cut out the segment of the gel containing the desired fragment of DNA.

14. Elute the DNA from the gel as described in Chapter 6, pages 6.22–6.31. The efficiency of the elution process can be checked with a hand-held minimonitor.

Alkaline agarose gels should first be neutralized by shaking for 45 minutes in 0.5 M Tris · Cl (pH 7.6) and then in 0.5 × TBE (see Appendix B) before the radiolabeled DNA is eluted.

The eluted DNA is ready for use as a probe and does not need to be further denatured.

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Isolation of Small (<150 Nucleotides) Probes by Alkaline Chromatography on Sepharose CL-4B

Probes less than 150 nucleotides in length may be isolated rapidly by chromatography through an alkaline column of Sepharose CL-4B.

1. Prepare a column (10 cm × 0.5 cm) of Sepharose CL-4B equilibrated in 0.1 N NaOH, 0.3 M NaCl, 0.001 M EDTA (pH 8.0). (See Appendix E for analogous procedure for a Sephadex column.)

2. Add 0.5 M EDTA (pH 8.0) to the sample to a final concentration of 10 mM (step 10, page 10.23).

3. Add 0.1 volume of 1 N NaOH to the sample, and incubate the mixture for 5 minutes at room temperature.

4. Load the sample onto the column and follow the progress of the radioactive material with a hand-held minimonitor.

5. Begin collecting samples when the radioactivity first begins eluting from the column. All of the radioactive probe should elute in a volume of approximately 0.8 ml.

6. Neutralize the solution by adding 0.5 volume of 2 M Tris · Cl (pH 8.0) and 1 volume of 0.1 N HCl.

7. Check the size of the probe by electrophoresis through a 5% polyacrylamide gel (see Chapter 6, pages 6.39–6.43).

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Troubleshooting

The most common problem with synthesis of single-stranded probes by primer extension is premature termination of newly synthesized DNA chains. This occurs for a variety of reasons, most of which originate with the radiolabeled dNTP, which is rate-limiting in the reaction. Obviously, if the single-stranded DNA synthesis does not extend to a restriction site either within or downstream from the region of interest, the probe cannot be recovered as a discrete band by gel electrophoresis. To minimize this problem:

- *Use fresh radioisotopes.* Radioactive dNTPs that have been thawed more than twice are incorporated less efficiently into DNA by DNA polymerases.
- *Use freshly made reaction mixture.* Dilute solutions of dNTPs are not stable at −20°C.
- *Keep the amount of template in the reaction to a minimum.* The greater the number of template molecules in the reaction, the shorter the length of the newly synthesized chains.
- *Design the bacteriophage M13 or phagemid recombinant so that the probe will be as short as possible.* If the average length of the DNA synthesized during the first stage of the reaction (i.e., before addition of the unlabeled dNTP) is less than the final length of the probe, the specific activity of the 5′ terminus of the probe will be considerably higher than that of the 3′ terminus. In general, this asymmetry does not matter when the probes are to be used in northern or Southern hybridization. However, it can vitiate attempts to use the probe in nuclease-S1 mapping of RNAs. This problem can be minimized by ensuring that the final length of the probe is less than the length of the DNA synthesized during the first stage of the reaction.

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SYNTHESIS OF RNA PROBES BY IN VITRO TRANSCRIPTION OF DOUBLE-STRANDED DNA TEMPLATES BY BACTERIOPHAGE DNA-DEPENDENT RNA POLYMERASES

The technical advance that made it possible to synthesize single-stranded RNA probes of high specific activity was the development of plasmid vectors containing polycloning sites downstream from powerful promoters derived from the *Salmonella typhimurium* bacteriophage SP6 (Green et al. 1983) or from the *E. coli* bacteriophages T7 and T3 (Studier and Rosenberg 1981; Davanloo et al. 1984; Tabor and Richardson 1985). These promoters were chosen because they are recognized specifically by DNA-dependent RNA polymerases encoded by their respective bacteriophages. Although all three of the enzymes share many biochemical properties (for review, see Chamberlin and Ryan 1982), each has a distinct specificity for its cognate promoter and does not use promoters recognized by other polymerases at any substantial rate. Furthermore, these bacteriophage enzymes do not recognize bacterial or plasmid promoters or eukaryotic promoters in cloned DNA sequences. In addition, they initiate RNA synthesis at aberrant sites at a very low rate, do not efficiently initiate transcription at nicks in double-stranded DNA templates, will transcribe homopolymeric tracts at least 30 nucleotides in length, and are able to transcribe long segments of cloned DNA in vitro. Thus, when a linearized plasmid is incubated in vitro with the appropriate DNA-dependent RNA polymerase and the four rNTPs, virtually all RNA synthesis is initiated at the bacteriophage promoter. The transcripts, which terminate at the end of the linear DNA molecule, are complementary to only one of the two strands of the template (see Figure 10.2, page 10.5). They can therefore be used as strand-specific reagents in a variety of different hybridization reactions. RNAs generated by in vitro transcription share many of the advantages of single-stranded DNA probes. For example, they can be synthesized to very high specific activity, and they hybridize to their targets far more efficiently than conventional nick-translated probes. However, RNA probes are superior to single-stranded DNA probes in several respects:

- Radiolabeled RNA can be produced much more efficiently than single-stranded DNA. Because the templates used to synthesize RNA can be transcribed many times, the yield of RNA can exceed the weight of the template severalfold.
- Bacteriophage DNA-dependent RNA polymerases function efficiently in vitro in the presence of relatively low concentrations of rNTPs (1–20 μM), so that full-length probes of high specific activity can be synthesized relatively inexpensively.
- RNA probes can be freed from template DNA merely by treating the reaction with RNAase-free DNAase I; they do not need to be purified by gel electrophoresis.
- Because of the innately higher stability of hybrids involving RNA (Casey and Davidson 1977), RNA probes generally yield stronger signals in hybridization reactions than do DNA probes of equal specific activity.

- Finally, the availability of RNA probes has led to improvements in the methods used to analyze transcripts of mammalian genes. Instead of digesting DNA:RNA hybrids with nuclease S1—an enzyme that can be difficult to control—RNA:RNA hybrids are digested with RNAase A, which can be used at a wide range of concentrations without compromising the results of the experiment (Zinn et al. 1983; Melton et al. 1984).

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Plasmid Vectors for Preparing RNA Probes

A large number of plasmids are available that carry a cloned copy of a bacteriophage promoter (derived from bacteriophage SP6, T7, or T3) adjacent to a polycloning site. The range of cloning sites in these vectors is now so large that foreign DNA sequences can usually be inserted by directional cloning no further than 30–40 nucleotides downstream from the position at which RNA synthesis is initiated in vitro. In many cases, the polycloning site also provides a restriction site downstream from the foreign DNA that can be used to linearize the recombinant plasmid. In addition, several vectors are available that contain two different bacteriophage promoters arranged in opposite orientations and separated by a polycloning site. Because of the very high specificity of the bacteriophage polymerases for their promoters, these vectors can be used to generate single-stranded probes complementary to either one strand or the other of the foreign DNA. Finally, more complex plasmids are available that, in addition to bacteriophage promoters, contain, for example, the region of the *E. coli lacZ* gene coding for the α-peptide of β-galactosidase and/or the origin of replication of a single-stranded bacteriophage. Insertion of foreign DNA sequences into the polycloning site of these vectors can be scored by the appearance of white colonies on plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The bacteriophage origin of replication can be activated by infection of F′ bacteria carrying recombinant plasmids with a helper bacteriophage (Vieira and Messing 1987). The resulting single-stranded DNA can be used as a template for sequencing or mutagenesis (Mead et al. 1986). (See Chapters 1 and 4 for more detailed discussion of specific plasmid and single-stranded, filamentous bacteriophage vectors.)

The choice between these vectors is largely a matter of personal preference. However, if transcripts of both strands of the template will be required, it would be more efficient to choose a plasmid carrying two different bacteriophage promoters than to insert the template in two orientations into a plasmid carrying a single promoter. It is also important to consider the disposition of restriction sites within the template DNA and downstream from it. The 5′ terminus of the transcript is fixed by the bacteriophage promoter, but the 3′ terminus is determined by the downstream site of cleavage by the restriction enzyme. Thus, by using different restriction enzymes to generate the linear DNA, a number of different probes can be prepared from a single recombinant plasmid. However, the presence of plasmid sequences in the probe can increase the level of background hybridization and is clearly unacceptable if the probe is to be used to screen a plasmid library. To minimize this problem, choose a vector with a suitable restriction site in the downstream region of the polycloning site. If possible, use a restriction enzyme that generates blunt ends or protruding 5′ termini. Transcription of templates with protruding 3′ termini results in the synthesis of significant amounts of long RNA molecules that are aberrantly initiated at the termini of the templates (Schenborn and Mierendorf 1985). If it is impossible to avoid using a restriction enzyme that creates protruding 3′ termini, treat the DNA with the Klenow fragment of *E. coli* DNA polymerase I or with bacteriophage T4 DNA polymerase (enzymes that exonucleolytically remove the protruding tail) before using it as a template.

Recently, bacteriophage promoters have been incorporated into bacteriophage λ and cosmid vectors. Usually, these vectors contain two different bacteriophage promoters arranged in opposite orientations on either side of the site into which foreign DNA is inserted (e.g., λZAP [see Chapter 2, page 2.53] and cosmid pWE [Wahl et al. 1987; see Chapter 3, page 3.21]). When a recombinant genome constructed in vectors of this type is digested with a restriction enzyme that cleaves many times within the foreign DNA, a large number of fragments will be generated, one of which contains a particular bacteriophage promoter and the foreign sequences that lie immediately downstream from it. If the fragments do not contain protruding 3′ termini, only the fragment carrying the bacteriophage promoter will serve as a template in an in vitro transcription reaction. The resulting radiolabeled RNA, which is complementary to sequences located at one end of the original segment of foreign DNA, can then be used as a probe to isolate overlapping clones from genomic DNA or cDNA libraries. These vectors therefore greatly simplify the task of "walking" from one recombinant clone to another along a chromosome.

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Preparation of DNA Template

The DNA used in the in vitro transcription reaction need not be highly purified—crude minipreparations work well. The essential requirement is that the template be free of RNAase contamination, a criterion that can usually be fulfilled by extracting the preparation with phenol:chloroform. Templates used for in vitro transcription either should be blunt-ended or should carry protruding 5′ termini. Protruding 3′ termini generated by cleavage with restriction enzymes such as *Pst*I or *Sst*I should be removed enzymatically using the Klenow fragment of *E. coli* DNA polymerase I or bacteriophage T4 DNA polymerase (see Appendix F).

It is essential that plasmid DNA templates be cleaved to completion, since trace amounts of supercoiled plasmid DNA will result in the generation of extremely long transcripts that include plasmid sequences. Because of their length, these transcripts can account for a substantial proportion of the incorporated radiolabeled rNTP.

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Synthesis of RNA In Vitro

The conditions for in vitro transcription of linearized plasmid DNA containing a bacteriophage promoter are:

40 mM Tris · Cl (pH 7.5)
6 mM $MgCl_2$
2 mM spermidine HCl
5 mM NaCl
10 mM dithiothreitol
100 μg/ml bovine serum albumin (Fraction V; Sigma)
20 nM linearized plasmid DNA (40 μg/ml of a 3-kb plasmid)
500 μM each rATP, rCTP, rUTP
2–20 μM [α-^{32}P]rGTP (sp. act. 400–3000 Ci/mmole)
1 unit/μl placental RNAase inhibitor
500–1000 units/ml bacteriophage DNA-dependent RNA polymerase

Nucleotide concentration. More than 90% of the transcripts are full-length when the concentration of each of the rNTPs is 50 μM or greater. Because bacteriophage SP6 DNA-dependent RNA polymerase is more tolerant of lower concentrations of rGTP than of the other three rNTPs, [α-^{32}P]rGTP is used as the radiolabeled nucleotide.

Spermidine and bovine serum albumin. When used in combination, these reagents stimulate transcription 2–2.5-fold.

Enzymes. The DNA-dependent RNA polymerases of bacteriophages T3, T7, and SP6 each consist of a single polypeptide chain (M_r ~95,000) and share many biochemical properties (Chamberlin and Ryan 1982). The polymerase of bacteriophage SP6 is slightly more stable and easier to prepare than the enzymes encoded by bacteriophages T3 and T7, but this is not a significant practical advantage under normal conditions. The effects of varying the concentrations of rNTPs, DNA template, and enzyme on the activity of bacteriophage SP6 DNA-dependent RNA polymerase in vitro are described in detail by Butler and Chamberlin (1982) and Melton et al. (1984). As far as is known, bacteriophages T3 and T7 DNA-dependent RNA polymerases behave in a similar fashion. When the concentration of template is 20 nM and the concentration of each of the rNTPs is 50 μM or greater, the rate of RNA synthesis is linear for at least an hour and is proportional to the amount of DNA-dependent RNA polymerase added to the reaction. Under these conditions, about 8 moles of transcript are generated per mole of template. The yield of RNA can often be doubled if a second aliquot of enzyme is added to the reaction after 1 hour.

Template concentration. The concentration of template in the reaction should be approximately 20 nM (equivalent to 40 μg/ml of a plasmid 3 kb in length).

Reagents used for in vitro transcription.

a. Linear template DNA (0.2 pmole/10-μl reaction; 0.4 μg of a 3-kb plasmid).

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b. Placental RNAase inhibitor (20 units/μl), which is available from several manufacturers.

c. DNA-dependent RNA polymerase of bacteriophage T3, T7, or SP6. This is supplied by several manufacturers at a concentration of 10–20 units/μl.

d. RNAase-free pancreatic DNAase I (1 mg/ml), which is available from several manufacturers. Alternatively, contaminating RNAase can be removed from preparations of DNAase I by the methods given in Appendix B.

e. Transcription buffer.

10 × Transcription buffer

400 mM Tris · Cl (pH 7.5 at 37°C)
60 mM $MgCl_2$
20 mM spermidine HCl
50 mM NaCl

This buffer is autoclaved and stored in aliquots at −20°C.

To avoid contamination with RNAases, the following solutions should be made up in water that has been treated with 0.1% diethyl pyrocarbonate (DEPC) for at least 12 hours at 37°C and autoclaved for 15 minutes at 15 lb/sq. in. on liquid cycle.

Caution: DEPC is suspected to be a carcinogen and should be handled with care.

f. Dithiothreitol (1 M).

g. Bovine serum albumin (2 mg/ml) (Fraction V; Sigma).

h. rNTPs (rATP, rCTP, rGTP, and rUTP). Each rNTP is made up separately at an estimated concentration of 20 mM. All four solutions should be adjusted to pH 7.0 by addition of small quantities of a dilute solution (0.05 M) of Tris base (see Appendix C). Using the 20 mM stock solutions, prepare a mixture of rATP, rCTP, and rUTP, each at a concentration of 5 mM.

Reserve a fresh, unopened bottle of Tris crystals for preparation of RNAase-free solutions.

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Synthesis of RNA Probes Radiolabeled to High Specific Activity

1. Prepare 2 pmoles of template DNA by digestion with a suitable restriction enzyme. Analyze an aliquot (100 ng) of the digested DNA by agarose gel electrophoresis. If necessary, add more restriction enzyme and continue incubation until there is no trace of the undigested DNA.

   1 pmole of a plasmid 3 kb in length weighs ~2 μg.

2. If necessary, remove protruding 3′ termini by treating the digested DNA with the Klenow fragment of *E. coli* DNA polymerase I or bacteriophage T4 DNA polymerase in the presence of all four dNTPs (see Appendix F).

3. Purify the template DNA by extraction with phenol:chloroform and precipitation with ethanol (see Appendix E). Then redissolve the DNA in TE (pH 7.6) at a concentration of 250–500 nM.

   If contamination of the template DNA with RNAase is likely to be a problem, e.g., when using minipreparations of plasmid DNA that have been treated with RNAase, purify the DNA at this stage as follows:
   a. Add 0.1 volume of 10× proteinase K buffer.

   10× Proteinase K buffer

   500 mM NaCl
   50 mM EDTA (pH 8.0)
   100 mM Tris·Cl (pH 8.0)

   b. Add 0.1 volume of 5% SDS.
   c. Add proteinase K (self-digested; 20 mg/ml [see Appendix B]) to a final concentration of 100 μg/ml. Incubate the reaction for 1 hour at 37°C.
   d. Purify the DNA by extraction with phenol:chloroform and precipitation with ethanol.
   e. Redissolve the DNA in RNAase-free water at a concentration of 250–500 nM.

4. In a microfuge tube, mix the following components, in the order given, at room temperature:

RNAase-free H_2O	0.4 μl
template DNA in a volume of 1 μl or less	0.2 pmole
1 M dithiothreitol	0.1 μl
a solution containing rATP, rCTP, and rUTP, each at a concentration of 5 mM	1.0 μl
10× transcription buffer (see page 10.33)	1.0 μl
placental RNAase inhibitor (10 units)	0.5 μl
2 mg/ml bovine serum albumin (Fraction V; Sigma)	0.5 μl
[α-^{32}P]rGTP (sp. act. 400–3000 Ci/mmole; 10 μCi/μl)	5.0 μl
bacteriophage DNA-dependent RNA polymerase (7–12 units)	1.0 μl

   Mix the reagents by gently tapping the outside of the tube. Try to avoid producing air bubbles (this is sometimes difficult because commercial

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preparations of bacteriophage DNA-dependent RNA polymerase contain detergents as stabilizers). If necessary, remove air bubbles by brief centrifugation.

Incubate the reaction for 1–2 hours at 37°C (bacteriophages T3 and T7 DNA-dependent RNA polymerases) or 40°C (bacteriophage SP6 DNA-dependent RNA polymerase).

The components are added in the order shown at room temperature to avoid the possibility that the template DNA may be precipitated by the high concentration of spermidine in the transcription buffer.

[α-^{32}P]rGTP is the radiolabel of choice because bacteriophage SP6 DNA-dependent RNA polymerase tolerates low concentrations of this rNTP slightly better than it tolerates low concentrations of any of the other three rNTPs. However, occasional batches of [α-^{32}P]rGTP (which is less stable than other radiolabeled rNTPs) give problems. If trouble should arise, try using [α-^{32}P]rUTP or obtain a fresh batch of [α-^{32}P]rGTP.

5. Add 1 μl of RNAase-free pancreatic DNAase I (1 mg/ml). Mix, and then incubate the reaction for 15 minutes at 37°C.

6. Add 100 μl of RNAase-free H_2O, and purify the RNA by extraction with phenol:chloroform.

7. Transfer the aqueous phase to a fresh tube, and add 20 μl of 5 M ammonium acetate. Mix, and then add 250 μl of ice-cold ethanol. After storage for 30 minutes at −20°C, collect the RNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge.

8. Remove as much of the ethanol as possible by gentle aspiration, and leave the open tube on the bench for a few minutes to allow the last visible traces of ethanol to evaporate. Redissolve the RNA in 100 μl of RNAase-free H_2O. Add 2 volumes of ice-cold ethanol and store at −70°C until needed.

   To recover the RNA, withdraw an aliquot of the ethanolic solution, add 0.1 volume of 5 M ammonium acetate, mix, and store the solution at −20°C for at least 15 minutes. Centrifuge the solution at 12,000*g* for 10 minutes at 4°C in a microfuge. Remove the ethanol, and dissolve the RNA in the desired volume of the appropriate RNAase-free buffer.

Notes

i. These conditions typically result in the incorporation of 80–90% of the radiolabel. The yield of RNA is ~40 pg (sp. act. $>10^9$ cpm/μg) when the specific activity of the [α-^{32}P]rGTP is 3000 Ci/mmole and ~0.2 μg (sp. act. $\sim 0.5 \times 10^8$ cpm/μg) when the specific activity of the precursor is 400 Ci/mmole.

ii. Two rounds of precipitation with ethanol should remove >99.0% of the oligonucleotides and unincorporated rNTPs from the RNA. Alternatively, the RNA may be purified by chromatography on a spun column of Sephadex G-50 (see Appendix E) that has been autoclaved in 10 mM Tris · Cl (pH 7.5).

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Troubleshooting

Problems that occasionally arise when using in vitro transcription systems include:

- *No RNA synthesis.* The most common cause of an apparent lack of RNA synthesis is contamination of tubes or reagents with RNAase. This can be avoided by taking the precautions described in Chapter 7, pages 7.3–7.4. Less frequently, failure to synthesize RNA is a consequence of precipitation of the DNA template by the spermidine in the 10 × transcription buffer. Make sure that the components of the reaction are assembled at room temperature and in the stated order. If necessary, the presence of soluble template can be confirmed by analyzing an aliquot of the reaction by electrophoresis through an agarose gel. Finally, as discussed above, occasional batches of radiolabeled rGTP work inefficiently as precursors for the synthesis of RNA. When synthesizing RNA of high specific activity, try to use fresh batches of [α-^{32}P]rGTP that have not been frozen and thawed many times. If you suspect that the radiolabeled rGTP is at fault, set up a test reaction containing another radiolabeled rNTP.

- *Transcription from the wrong strand of DNA.* Usually less than 0.2% of transcripts synthesized in vitro by bacteriophage DNA-dependent RNA polymerases are derived from the complementary DNA strand (Melton et al. 1984). However, this high degree of specificity is only achieved with linear templates that do not carry protruding 3′ termini. Contamination of the template with superhelical plasmid DNA will cause an increase in aberrant initiation of RNA chains (on both strands of the DNA), and the presence of a protruding 3′ terminus downstream from the bacteriophage promoter will lead to the synthesis of transcripts complementary to the full length of the wrong strand of DNA. Both of these problems can be avoided by careful preparation of the template.

- *Synthesis of transcripts that are less than the desired length.* The synthesis of less-than-full-length transcripts can be due to (1) the fortuitous occurrence in the template of sequences that terminate transcription by the particular bacteriophage DNA-dependent RNA polymerase used and/or (2) limiting concentrations of precursor (usually the radiolabeled rNTP).

  The first of these problems can sometimes be cured by constructing a new plasmid in which transcription of the sequences of interest is driven by a polymerase encoded by a different bacteriophage. Some sequences recognized as terminators by one bacteriophage DNA-dependent RNA polymerase are not recognized as efficiently by another. It may therefore be worthwhile to compare the lengths of the RNAs produced when the sequences of interest are transcribed from the appropriate promoter by different bacteriophage DNA-dependent RNA polymerases.

  A number of steps can be taken to minimize premature termination of nascent RNA chains:

  a. Increase the concentration of the limiting rNTP. Although the strengths of transcription termination signals vary greatly, all but the strongest of them can be overcome, at least in part, by increasing the concentration

of the limiting rNTP. In most cases, it is impractical to increase the concentration of the radiolabeled rNTP in the reaction, since the improvement in yield of full-length product is gained at the expense of reducing the specific activity of the probe.

b. Lower the temperature of incubation of the transcription reaction to 30°C. This has been shown in some cases to increase the proportion of full-length transcripts (Krieg and Melton 1987).

c. Pare the sequences to be transcribed to the minimum. In this way, it may be possible to eliminate the termination sequences from the clone.

d. Purify the desired product by electrophoresis through a polyacrylamide or agarose gel. The transcription reactions are so efficient that it is often possible to purify sufficient quantities of the desired RNA, even if it is only a relatively minor proportion of the total RNA synthesized in the reaction.

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SYNTHESIS OF cDNA PROBES

cDNA probes are used chiefly to isolate cDNA clones corresponding to genes that are expressed more actively in one type of cell than in another. In this class are genes that are developmentally regulated and genes that are specifically expressed in particular types of differentiated cells. The identification of cDNA clones may be accomplished by a number of methods, all of which are capable of detecting differences in gene expression between closely related types of cells.

This section presents methods to generate:

- Total cDNA probes
- Radiolabeled subtracted cDNA probes
- Subtracted cDNA probes radiolabeled to high specific activity

Identification of cDNA Clones

DIFFERENTIAL SCREENING

The simplest form of differential screening is shown in Figure 10.3. Poly(A)$^+$ RNAs extracted from two cell types are used as templates to generate radiolabeled cDNA probes that are hybridized separately to duplicate copies of the same cDNA library. Clones that hybridize to both probes correspond to genes that are expressed in both cell types, whereas clones that hybridize to only one probe correspond to mRNAs that are expressed in only one cell type. In principle, this type of comparative screening can be used to identify any differentially regulated gene. In practice, however, the method works well only for the small subclass of such genes that are expressed in abundance in one of the two types of cells. Examples of cDNAs that have been isolated in this way include those corresponding to genes that are expressed at different times during development of *Drosophila* (Scherer et al. 1981) and *Dictyostelium* (Mehdy et al. 1983); human genes whose activities are markedly induced by estradiol (Masiakowski et al. 1982); murine genes that are expressed at high levels in cells transformed by SV40 (Schutzbank et al. 1982); genes that are expressed in growing, but not quiescent, mammalian cells (Foster et al. 1982; Linzer and Nathans 1983); genes whose mRNAs are localized to different regions of *Xenopus* eggs (Weeks et al. 1985); and genes whose activities are induced in the livers of rats that have been exposed to phenobarbital (Lechner et al. 1987).

Because they are copied from the entire population of mRNAs, cDNA probes used in differential screening are of very high complexity, with each mRNA represented in the probe approximately in proportion to its abundance in the population. Since most animal cells contain more than 10,000 different cytoplasmic mRNAs (Davidson 1986), the absolute amount of radioactivity in the probe complementary to an individual species of rare mRNA is extremely small—too small, in fact, to allow the detection of the corresponding cDNA by hybridization. Plus-minus screening is therefore reliable only when the mRNA of interest comprises more than about 0.05% of the total mRNA in one cell and less than 0.01% in the other. Because of this limitation, several

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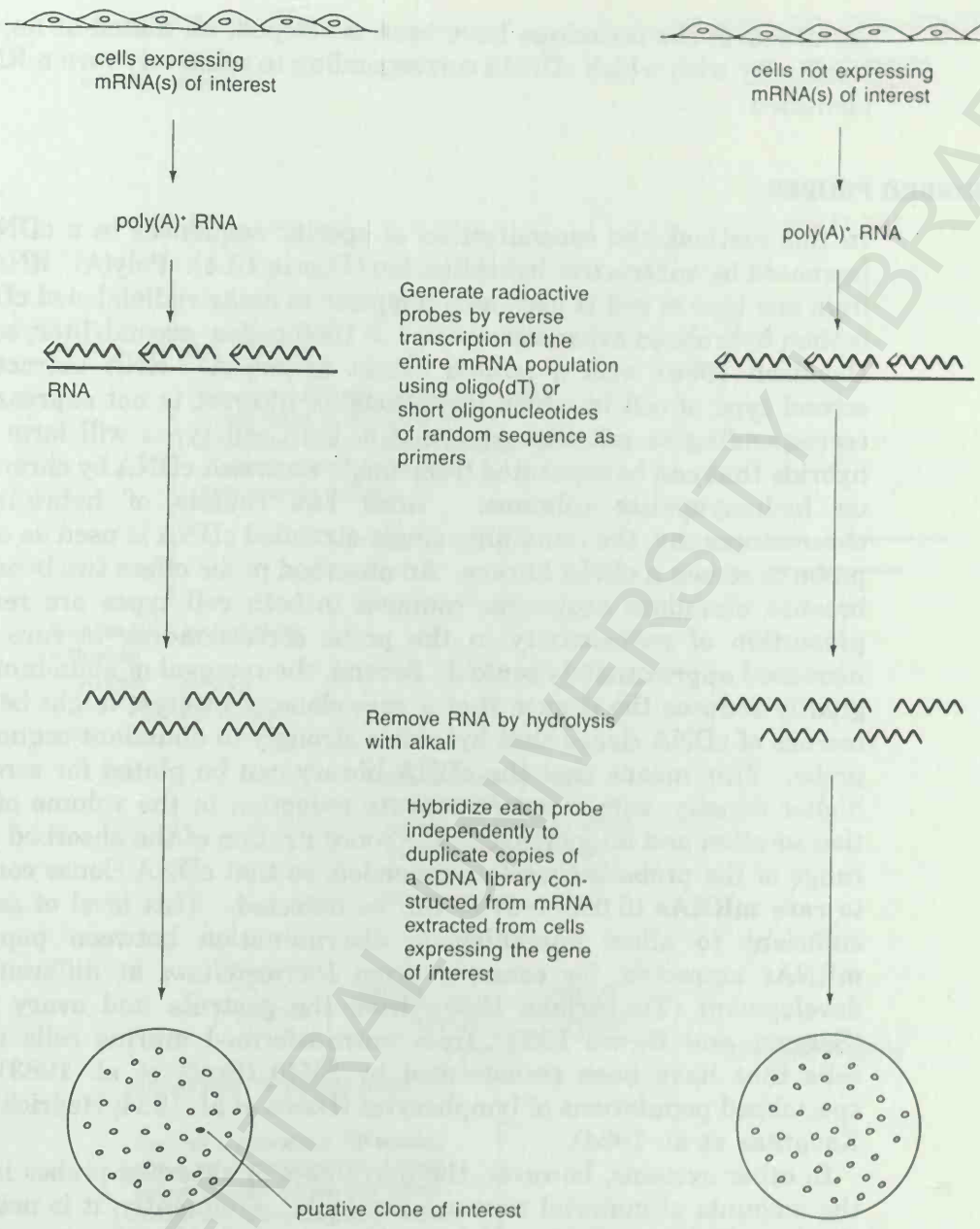

FIGURE 10.3
Differential screening.

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variations of the technique have been developed, all aimed at improving the sensitivity with which cDNAs corresponding to relatively rare mRNAs can be identified.

ABSORBED PROBES

In this method, the concentration of specific sequences in a cDNA probe is increased by subtractive hybridization (Figure 10.4). Poly(A)$^+$ RNA extracted from one type of cell is used as a template to make radiolabeled cDNA, which is then hybridized exhaustively ($C_{r_0}t > 1000$ moles · second/liter; see p. 538 in Davidson 1986) with a tenfold excess of poly(A)$^+$ RNA extracted from a second type of cell in which the gene(s) of interest is not expressed. cDNAs corresponding to mRNAs expressed in both cell types will form DNA:RNA hybrids that can be separated from single-stranded cDNA by chromatography on hydroxyapatite columns. After two rounds of hybridization and chromatography, the remaining single-stranded cDNA is used as an absorbed probe to screen a cDNA library. An absorbed probe offers two benefits. First, because abundant sequences common to both cell types are removed, the proportion of radioactivity in the probe corresponding to rare mRNAs is increased approximately tenfold. Second, the removal of abundant sequences greatly reduces the chance that a rare clone of interest might be lost in the morass of cDNA clones that hybridize strongly to abundant sequences in the probe. This means that the cDNA library can be plated for screening at a higher density, with a commensurate reduction in the volume of hybridization solution and an increase in the concentration of the absorbed probe. The range of the probe is therefore extended, so that cDNA clones corresponding to rare mRNAs (0.005–0.01%) can be detected. This level of sensitivity is sufficient to allow unambiguous discrimination between populations of mRNAs extracted, for example, from *Dictyostelium* at different stages of development (Timberlake 1980), from the gastrula and ovary of *Xenopus* (Sargent and Dawid 1983), from untransformed murine cells and murine cells that have been transformed by SV40 (Scott et al. 1983), and from specialized populations of lymphocytes (Davis et al. 1984; Hedrick et al. 1984; Kavathas et al. 1984).

In other systems, however, the usefulness of absorbed probes is limited by the amounts of material that are available. Frequently, it is not possible to obtain sufficient mRNA to drive subtractive hybridization to completion; in other circumstances, because the amount of radioactivity in an absorbed probe that is complementary to any given cDNA is small, hybridization to a subtracted library may be inefficient, and satisfactory signals may be obtained only after prolonged exposure of autoradiographs. In either case, the probability of isolating a cDNA corresponding to a very rare mRNA is obviously remote.

SUBTRACTED LIBRARIES

Some of the problems of absorbed probes can be circumvented by constructing subtracted libraries (Figure 10.5). Poly(A)$^+$ RNA extracted from one type of cell is used as a template to make cDNA, which is then annealed to poly(A)$^+$

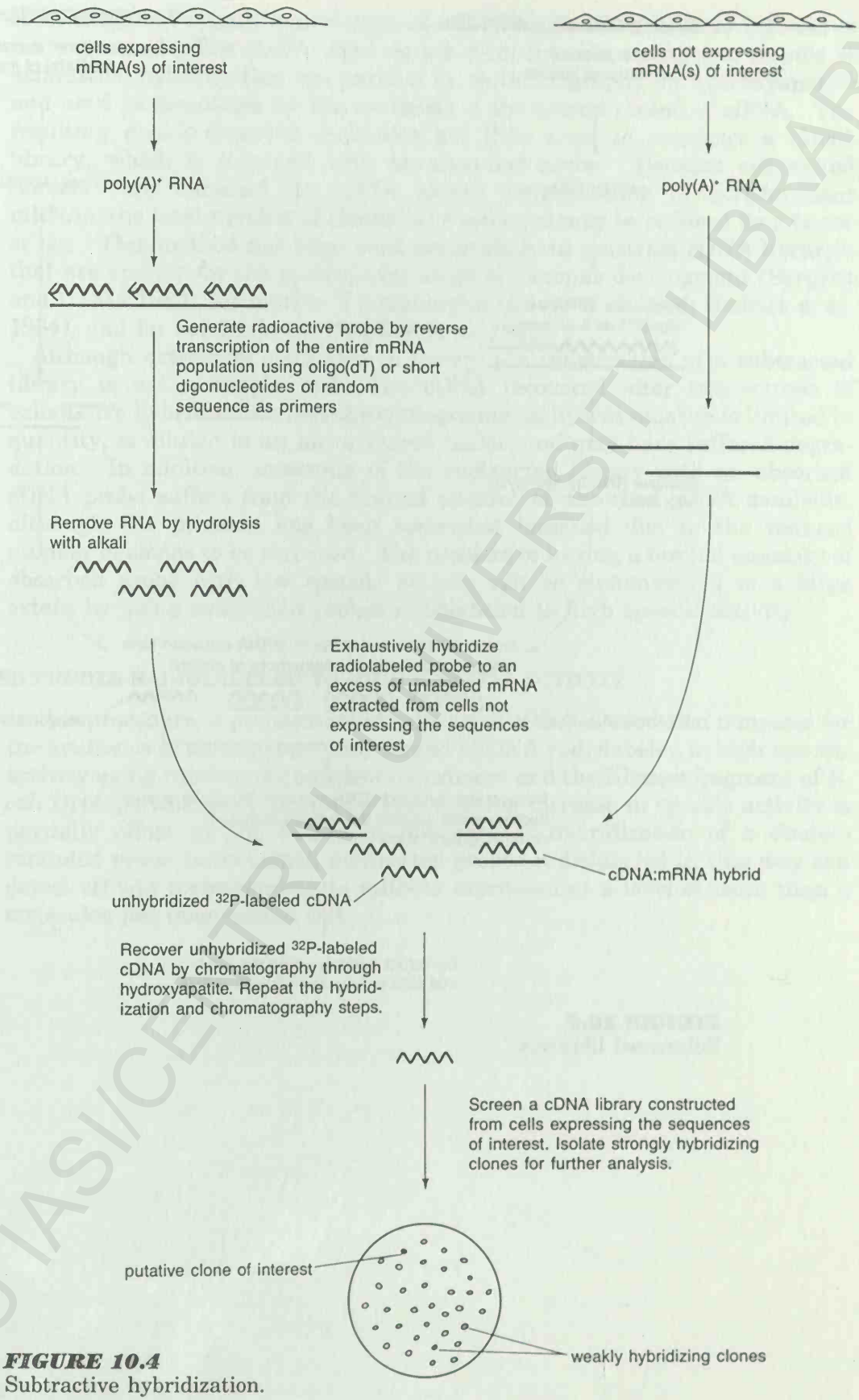
cells expressing mRNA(s) of interest
cells not expressing mRNA(s) of interest
poly(A)+ RNA
poly(A)+ RNA
Generate radioactive probe by reverse transcription of the entire mRNA population using oligo(dT) or short digonucleotides of random sequence as primers
Remove RNA by hydrolysis with alkali
Exhaustively hybridize radiolabeled probe to an excess of unlabeled mRNA extracted from cells not expressing the sequences of interest
cDNA:mRNA hybrid
unhybridized 32P-labeled cDNA
Recover unhybridized 32P-labeled cDNA by chromatography through hydroxyapatite. Repeat the hybridization and chromatography steps.
Screen a cDNA library constructed from cells expressing the sequences of interest. Isolate strongly hybridizing clones for further analysis.
putative clone of interest
weakly hybridizing clones

FIGURE 10.4
Subtractive hybridization.

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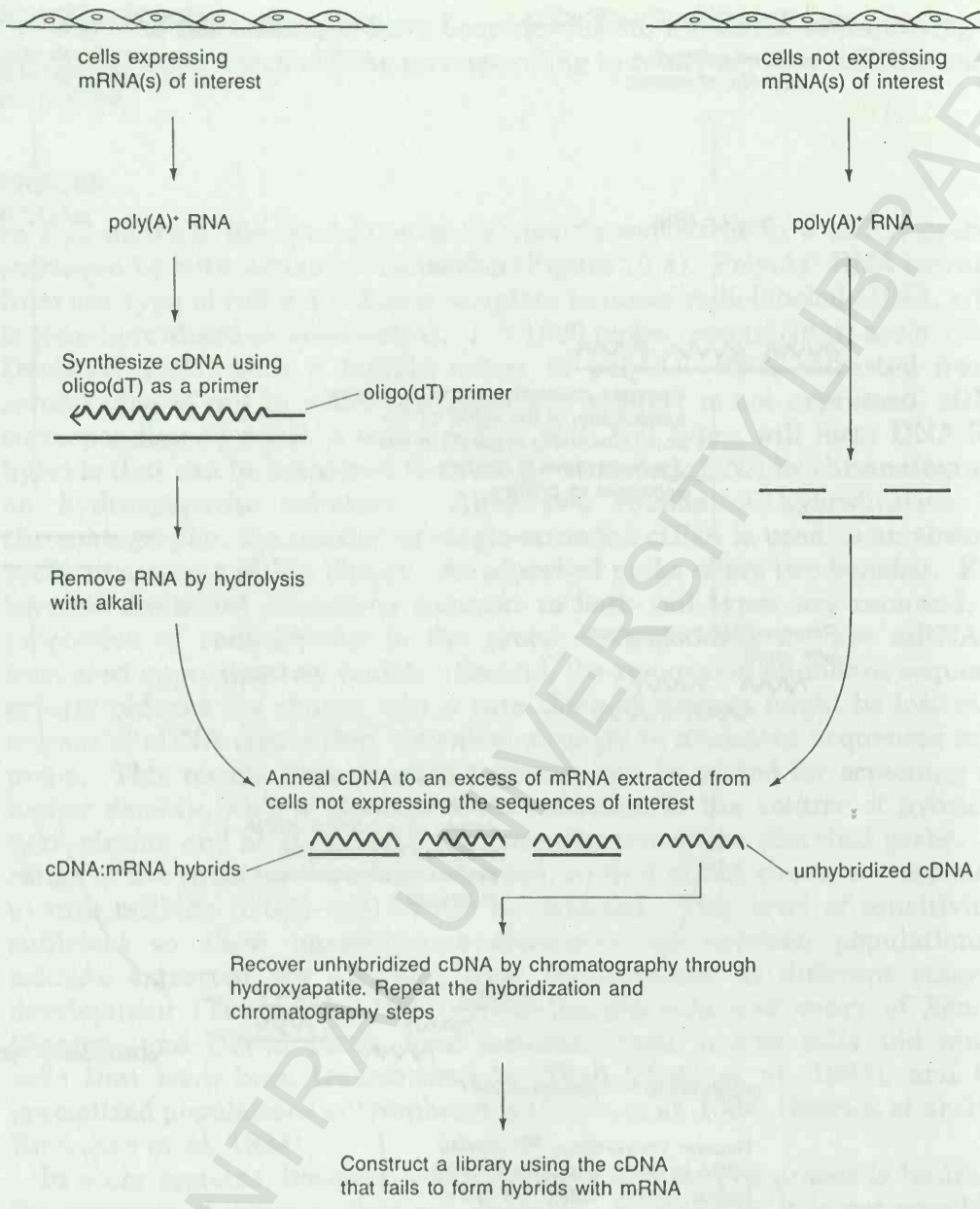

FIGURE 10.5
Subtracted libraries.

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RNA extracted from a second type of cell in which the gene(s) of interest is not expressed. The cDNAs that do not form hybrids during two rounds of exhaustive hybridization are purified by chromatography on hydroxyapatite and used as templates for the synthesis of the second strand of cDNA. The resulting double-stranded molecules are then used to construct a cDNA library, which is screened with an absorbed probe. Because subtracted libraries are enriched for cDNA clones corresponding to nonabundant mRNAs, the total number of clones to be screened may be reduced by a factor of ten. This method has been used successfully to construct cDNA libraries that are specific for the gastrulation stage of *Xenopus* development (Sargent and Dawid 1983), for murine T lymphocytes (Davis et al. 1984; Hedrick et al. 1984), and for rat forebrain (Rhyner et al. 1986).

Although extremely attractive in theory, the construction of a subtracted library is not a simple task: The cDNA recovered after two rounds of exhaustive hybridization and chromatography on hydroxyapatite is limited in quantity, is diluted in an inconvenient buffer, and may have suffered degradation. In addition, screening of the subtracted library with an absorbed cDNA probe suffers from the limited amount of absorbed cDNA available, although the problem has been somewhat lessened due to the reduced number of clones to be screened. The problem of having a limited quantity of absorbed probe with low specific activity can be circumvented to a large extent by using subtracted probes radiolabeled to high specific activity.

SUBTRACTED PROBES RADIOLABELED TO HIGH SPECIFIC ACTIVITY

In this procedure, a preparation of subtracted cDNA is used as a template for the synthesis of a complementary strand of DNA radiolabeled to high specific activity using random oligonucleotide primers and the Klenow fragment of *E. coli* DNA polymerase I. Although the resulting increase in specific activity is partially offset by the decreased efficiency of hybridization of a double-stranded probe to its target, subtracted probes radiolabeled in this way can detect cDNAs corresponding to mRNAs expressed at a level of more than 5 molecules per mammalian cell.

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Synthesis of Total cDNA Probes Complementary to Single-stranded RNA Using Oligonucleotides as Primers

^{32}P-labeled DNA probes that are complementary to mRNA templates may be synthesized using either oligo(dT) primers that bind to the poly(A) tract at the 3′ terminus of most mammalian mRNAs or random primers that are complementary to sequences distributed along the entire length of the mRNAs. The first method has two disadvantages: It can only be used with RNAs that carry poly(A) tracts, and it generates biased probes that contain disproportionate amounts of sequences derived from the 3′ terminus of the mRNAs. The second method, which avoids both of these problems, is similar in principle to that used to generate probes from double-stranded DNA templates (see pages 10.14–10.15), except that the polymerization reaction is catalyzed by RNA-dependent DNA polymerase (reverse transcriptase) rather than by the Klenow fragment of *E. coli* DNA polymerase I. As discussed above, because the template usually consists of a heterogeneous population of RNA molecules (e.g., a preparation of mRNA), the sequence complexity of the resulting probes is generally much greater than those generated from templates of cloned DNA. The proportion of radioactivity incorporated into the sequences of interest may therefore vary widely, depending on their abundance in the preparation of mRNA. To obtain sufficient radiolabeled DNA to serve as a probe for such rare sequences, it may therefore be necessary to use up to 10 μg of total mRNA as a template or to enrich the preparation for sequences of interest (see Chapter 8, pages 8.6–8.10). If the probe is to be used to screen cDNA or genomic DNA libraries, it should contain at least 10^4 cpm in sequences of interest.

1. In a sterile microfuge tube, mix 1 μg of RNA template with 3 μl of RNAase-free H_2O (see page 10.33). Heat the mixture to 70°C for 5 minutes, and then chill it quickly in ice water.

2. Add:

placental RNAase inhibitor	20 units
synthetic random octanucleotide primers (2.5 mg/ml)	5.0 μl
10× random primer buffer	2.5 μl
a solution of dGTP, dATP, and dTTP, each at a concentration of 20 mM	1.0 μl
120 μM dCTP	1.0 μl
[α-^{32}P]dCTP (sp. act. >3000 Ci/mmole; 10 μCi/μl)	10.0 μl
murine reverse transcriptase (Pharmacia, 200,000 units/ml)	0.5 μl
H_2O to 25 μl	

To scale this reaction up, increase the volumes of all components proportionally.

For a discussion of the disparity in the number of units of murine reverse transcriptase recommended by different manufacturers, see Chapter 8, page 8.58.

Mix the components by gentle vortexing, trying to avoid producing bubbles. This is sometimes difficult because reverse transcriptase is

supplied in a buffer containing Triton X-100. Bubbles may be removed by brief centrifugation in a microfuge.

Incubate the reaction for 1 hour at 37°C.

10 × Random primer buffer

0.5 M Tris · Cl (pH 7.6)
20 mM dithiothreitol
50 mM $MgCl_2$
0.4 M KCl

3. Add:

0.5 M EDTA (pH 8.0)	1 μl
10% SDS	1 μl

Mix well.

4. Add 3 μl of 3 N NaOH. Incubate the mixture for 30 minutes at 68°C to hydrolyze the RNA.

5. Cool the mixture to room temperature. Add 10 μl of 1 M Tris · Cl (pH 7.4). Mix well, and add 3 μl of 2 N HCl.

6. Purify the cDNA by extraction with phenol:chloroform (see Appendix E).

7. Separate the radiolabeled probe from the unincorporated dNTPs by

- chromatography on, or centrifugation through, a small column of Sephadex G-50 (see Appendix E) or
- selective precipitation of labeled DNA by ethanol (see Appendix E).

Notes

i. Approximately 30% of the [α-^{32}P]dCTP should have been incorporated into cDNA. Incubating the reaction for longer periods of time does not usually result in the incorporation of more radiolabel because murine reverse transcriptase has a short half-life at 37°C.

ii. When probing a cDNA library, use 2×10^7 to 5×10^7 cpm of radiolabeled cDNA for each 138-mm filter and 7×10^6 to 1.5×10^7 cpm for each 82-mm filter. The radiolabeled cDNA can be used for hybridization without denaturation.

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Synthesis of Radiolabeled, Subtracted cDNA Probes Using Oligo(dT) as a Primer

1. Carry out a large-scale synthesis of cDNA (see steps 1–6, pages 10.44–10.45) using 5–10 μg of RNA as template and 0.5–1.0 mCi of radiolabeled dCTP. This requires that the volume of all other components in the reaction be increased by a factor of 5–10 and that 1 μg of oligo(dT)$_{12-18}$ for each microgram of mRNA be substituted for the random primers.

   Measure the total and trichloroacetic acid (TCA)-precipitable radioactivities in 1 μl of the reaction (see Appendix E). Calculate the yield of cDNA as follows. In a reaction containing 1.5 nmoles of the limiting dNTP:

   $$\frac{\text{cpm incorporated}}{\text{total cpm}} \times 2 = \mu\text{g of cDNA synthesized}$$

2. Purify the DNA by chromatography on a spun column of Sephadex G-50 as described in Appendix E.

   Important: All subsequent steps should be carried out in siliconized tubes (see Appendix E).

3. To the radiolabeled DNA, add a tenfold excess by weight of the RNA that will be used to subtract the cDNA probe, 0.2 volume of 5 M ammonium acetate, and 2.5 volumes of ice-cold ethanol. Store at 0°C for 10–15 minutes, and then recover the nucleic acids by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

4. Remove all of the ethanol by aspiration, and allow the pellet of nucleic acids to dry at room temperature. Redissolve the nucleic acids in 6 μl of RNAase-free H_2O (see page 10.33).

5. Add:

2 M sodium phosphate (pH 6.8)	2.0 μl
0.5 M EDTA (pH 8.0)	0.1 μl
20% SDS	0.1 μl

   2 M sodium phosphate (pH 6.8) is made by mixing equal quantities of 2 M Na_2HPO_4 and 2 M NaH_2PO_4.

6. Draw the mixture into a siliconized, disposable 20-μl glass capillary tube. Seal the ends of the tube in the flame of a Bunsen burner.

7. Place the sealed capillary tube in a boiling-water bath for 5 minutes. Transfer the tube to a water bath set at 68°C, and allow the nucleic acids to hybridize to $C_{r_o}t = 1000$ moles · seconds/liter.

   The rate of hybridization of the cDNA in the presence of excess RNA is approximated by the pseudo-first-order rate equation (see p. 538 in Davidson 1986):

   $$\frac{D}{D_o} = e^{-kC_{r_o}t}$$

   where D_o = initial concentration of cDNA, D = concentration of unreacted DNA at

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time t, k = observed rate constant for formation of RNA:DNA hybrids, and C_{r_o} = initial concentration of RNA (which does not change appreciably during the reaction).

8. Using forceps, remove the capillary tube from the water bath. Open the ends of the capillary tube with a file, and transfer the hybridization mixture into a tube containing 1 ml of SS buffer.

SS buffer

0.12 M sodium phosphate (pH 6.8)
0.1% SDS

9. Separate the single-stranded and double-stranded nucleic acids by chromatography on hydroxyapatite at 60°C as described in Appendix E. Measure the amount of radioactivity in each fraction.

   At least 90% of the cDNA should have hybridized to the mRNA.

10. Pool the fractions containing the single-stranded cDNA, and add an equal volume of 2-butanol. Mix the two phases by vortexing, and then centrifuge the mixture at 12,000*g* for 2 minutes at room temperature in a microfuge. Discard the upper (organic) phase.

11. Repeat the extraction with 2-butanol until the volume of the aqueous phase is < 100 μl.

12. Remove salts from the cDNA by chromatography on, or centrifugation through, a small column of Sephadex G-50 (see Appendix E).

13. Measure the amount of radioactivity, and calculate the weight of the cDNA recovered. Repeat steps 3–9.

Notes

i. Between 10% and 30% of the cDNA forms hybrids with the subtracted RNA during the second round of hybridization.

ii. It is not necessary to concentrate or remove salts from the final preparation of cDNA if it is to be used to probe a cDNA library.

iii. Probes radiolabeled to high specific activity are rapidly damaged by radiochemical decay. The subtractive hybridizations should be carried out as rapidly as practicable, and the probe should be used without delay.

iv. When probing a cDNA library, use 2×10^7 to 5×10^7 cpm of radiolabeled cDNA for each 138-mm filter and 7×10^6 to 1.5×10^7 cpm for each 82-mm filter. The radiolabeled cDNA can be used for hybridization without denaturation.

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This method works best when the cDNA is long. cDNAs synthesized using oligonucleotides of random sequence as primers tend to be shorter than cDNAs primed by oligo(dT). The cDNA used in this method is therefore synthesized using oligo(dT) as a primer under conditions that will favor the production of full-length copies of the mRNA templates (e.g., high concentrations of dNTPs).

1. Mix at 4°C in a sterile microfuge tube:

poly(A)$^+$ RNA (1 mg/ml)	10.0 μl
oligo(dT)$_{12-18}$ (1 mg/ml)	10.0 μl
1 M Tris · Cl (pH 7.6)	2.5 μl
1 M KCl	3.5 μl
250 mM $MgCl_2$	2.0 μl
mixture of all four dNTPs, each at a concentration of 5 mM	10.0 μl
[α-^{32}P]dCTP (sp. act. 3000 Ci/mmole; 10 μCi/μl)	5.0 μl
0.1 M dithiothreitol	2.0 μl
placental RNAase inhibitor	25 units
H_2O to 48 μl	

Add 2 μl of murine reverse transcriptase (Pharmacia, 200,000 units/ml). Mix well by gentle vortexing. Try to avoid producing bubbles. This is sometimes difficult because reverse transcriptase is supplied in a buffer containing Triton X-100. If necessary, bubbles may be removed by brief centrifugation in a microfuge.

Incubate the reaction for 2 hours at 37°C.

[α-^{32}P]dCTP is used as a tracer to measure the synthesis of the first strand of cDNA. Measure the total and trichloroacetic acid (TCA)-precipitable radioactivities in 1 μl of the reaction (see Appendix E). Calculate the yield of cDNA synthesized as follows. In a reaction containing 50 nmoles of each dNTP:

$$\frac{\text{cpm incorporated}}{\text{total cpm}} \times 66 = \mu\text{g of cDNA synthesized}$$

For a discussion of the disparity in the number of units of murine reverse transcriptase recommended by different manufacturers, see Chapter 8, page 8.58.

2. Stop the reaction by adding:

0.5 M EDTA (pH 8.0)	2 μl
10% SDS	2 μl

Mix well.

3. Remove the RNA by alkaline hydrolysis (steps 4–6, page 10.45), and purify the DNA by chromatography on a spun column of Sephadex G-50 as described in Appendix E.

4. Carry out two rounds of subtractive hybridization as described in steps 3–13 on pages 10.46–10.47.

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5. Concentrate the final preparation of cDNA by sequential extractions with 2-butanol, and remove salts by chromatography on Sephadex G-50 as described in steps 10–12, page 10.47.

6. Precipitate the cDNA with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ice-cold ethanol. Store at 0°C for 5–10 minutes, and recover the nucleic acids by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Rinse the pellet with 70% ice-cold ethanol. Carefully remove all of the ethanol, and allow the pellet to dry briefly at room temperature. Redissolve the DNA in water at a concentration of 15 ng/μl.

7. Mix:

subtracted cDNA	5 μl
synthetic random octanucleotide primers (2.5 mg/ml)	5 μl

8. Heat the mixture to 60°C for 5 minutes, and then cool it to 4°C.

9. Add:

100 mM dithiothreitol	5.0 μl
10 × RP buffer	5.0 μl
a solution containing dCTP, dGTP, and dTTP, each at a concentration of 5 mM	5.0 μl
[α^{-32}P]dATP (sp. act. > 3000 Ci/mmole; 10 μCi/μl)	25.0 μl
Klenow fragment of *E. coli* DNA polymerase I (12.5 units)	2.5 μl

Incubate the reaction for 4–6 hours at room temperature.

10 × RP buffer

900 mM HEPES (adjusted to pH 6.6 with 4 N NaOH)
100 mM $MgCl_2$

10. Add:

0.5 M EDTA (pH 8.0)	1.0 μl
20% SDS	2.5 μl

11. Separate the radiolabeled cDNA from the unincorporated dNTPs by chromatography on, or centrifugation through, a small column of Sephadex G-50 (see Appendix E).

Notes

i. The radiolabeled cDNA should be denatured by heating to 100°C for 5 minutes before it is used for hybridization.

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ii. When probing a cDNA library, use 5×10^7 cpm of radiolabeled cDNA for each 138-mm filter and 1.5×10^7 cpm for each 82-mm filter.

iii. Probes radiolabeled to high specific activity are rapidly damaged by radiochemical decay and should be used without delay.

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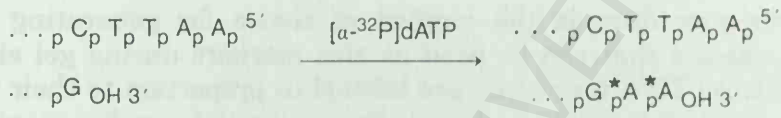
Labeling the 5′ and 3′ Termini of DNA

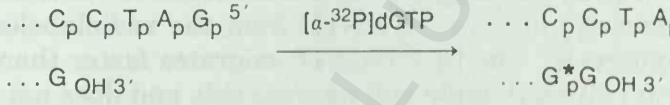
The termini of DNA molecules can be radiolabeled enzymatically to yield molecules that may be used in nuclease-S1 mapping of RNA, as radiolabeled primers in primer-extension reactions, and as markers of defined size in gel electrophoresis.

LABELING THE 3′ TERMINI OF DOUBLE-STRANDED DNA USING THE KLENOW FRAGMENT OF *E. coli* DNA POLYMERASE I

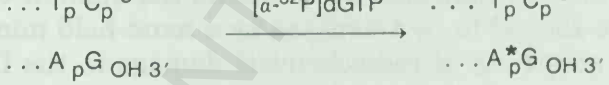
The reaction conditions are similar to those used to fill the recessed 3′ termini of double-stranded DNA molecules, except that only one radiolabeled dNTP is added to the reaction. Which of the four [α-^{32}P]dNTPs is added to the reaction depends on the sequence of the protruding 5′ termini at the ends of the DNA. For example, ends created by cleavage of DNA with *Eco*RI can be labeled with [α-^{32}P]dATP.



Termini created by cleavage of DNA with *Bam*HI can be labeled with [α-^{32}P]dGTP.



Blunt-ended DNA fragments are labeled by replacement of the nucleotide present at the 3′-hydroxyl terminus.



Unlabeled dNTPs are usually included in the reaction. This serves two purposes: It removes the possibility of exonucleolytic removal of nucleotides from the 3′ terminus of the template, and it allows the radiolabeled dNTP to be the second, third, or fourth nucleotide added to the recessed 3′ terminus. (DNA termini with 3′ extensions are not labeled efficiently by the Klenow fragment of *E. coli* DNA polymerase I. To label these molecules, use bacteriophage T4 DNA polymerase [see pages 10.54–10.55].)

The reaction may be carried out immediately after digesting the DNA with a restriction enzyme. There is no need to remove the restriction enzyme or to inactivate it. Because the Klenow fragment of *E. coli* DNA polymerase I works well under a variety of conditions, it is unnecessary to change buffers.

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1. Digest up to 1 μg of DNA with the desired restriction enzyme in 25 μl of the appropriate restriction enzyme buffer.

2. Add 2–10 μCi (sp. act. 400–3000 Ci/mmole) of the appropriate [α-^{32}P]dNTP.

3. Add 1 unit of the Klenow fragment of *E. coli* DNA polymerase I, and incubate the reaction for 15 minutes at room temperature.

4. Stop the reaction by heating it for 5 minutes at 70°C.

5. Separate the labeled DNA from unincorporated dNTP by

   - chromatography on, or centrifugation through, small columns of Sephadex G-50 (see Appendix E) or

   - two rounds of precipitation with ethanol (see Appendix E).

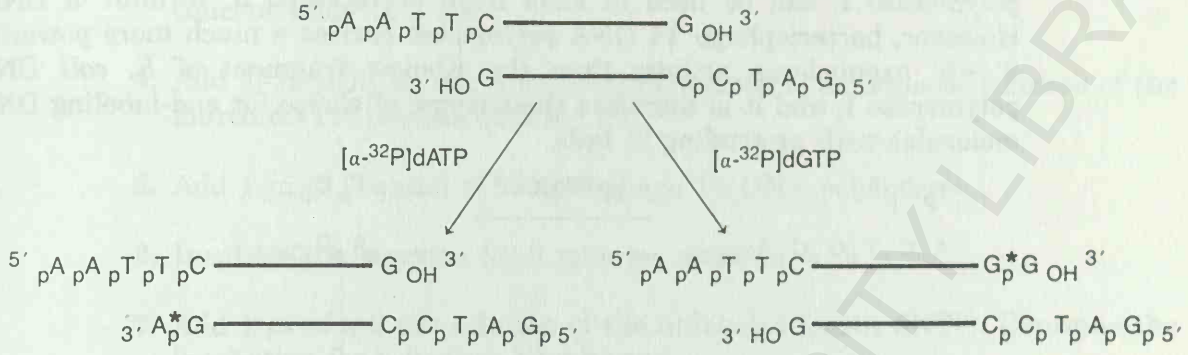
Notes

i. This procedure is the method of choice for generating labeled DNA fragments that can be used as size markers during gel electrophoresis. Because DNA fragments are labeled in proportion to their molar concentrations and not their sizes, both small and large fragments in a restriction enzyme digest become labeled to an equal extent. It is therefore possible to use autoradiography to locate bands of DNA that are too small to be visualized by staining with ethidium bromide. It is not necessary to remove unincorporated [α-^{32}P]dNTP from the radiolabeled DNA before gel electrophoresis. The [α-^{32}P]dNTP migrates faster than bromophenol blue on both polyacrylamide and agarose gels and does not interfere with the detection of anything but the smallest of DNA fragments. However, removing the unincorporated radiolabel has some advantages: It avoids the possibility of contaminating the anodic buffer chamber of the electrophoresis tank with radioactivity, it allows the amount of radiolabeled DNA applied to the gel to be estimated by a hand-held minimonitor, and it reduces the possibility of radiochemical damage to the DNA.

ii. The procedure works well on relatively crude DNA preparations (e.g., minipreparations of plasmids).

iii. When the labeled DNA is to be used for sequencing by the Maxam-Gilbert technique (see Chapter 13) or for mapping mRNA by the nuclease-S1 method (see Chapter 7), the concentration of labeled dNTP in the reaction should be increased to the greatest level that is practicable. After the reaction has been allowed to proceed for 15 minutes at room temperature, 2 nmoles of each of the four unlabeled dNTPs should be added (i.e., 1 μl of a 2 mM solution), and the incubation should be continued for a further 5 minutes at room temperature. This cold chase ensures that each recessed 3′ terminus will be completely filled and that all labeled DNA molecules will be exactly the same length.

iv. By choosing the appropriate [α-^{32}P]dNTP, it is possible to label only one end of a double-stranded DNA molecule. If, for example, the DNA has been cleaved by *Hin*dIII at one end and *Bam*HI at the other, it can be labeled selectively by including either [α-^{32}P]dATP or [α-^{32}P]dGTP in the reaction.


5′ pA pA pT pT pC — G OH 3′
3′ HO G — Cp Cp Tp Ap Gp 5′
[α-32P]dATP
[α-32P]dGTP
5′ pA pA pT pT pC — G OH 3′
3′ A*p G — Cp Cp Tp Ap Gp 5′
5′ pA pA pT pT pC — G*p G OH 3′
3′ HO G — Cp Cp Tp Ap Gp 5′

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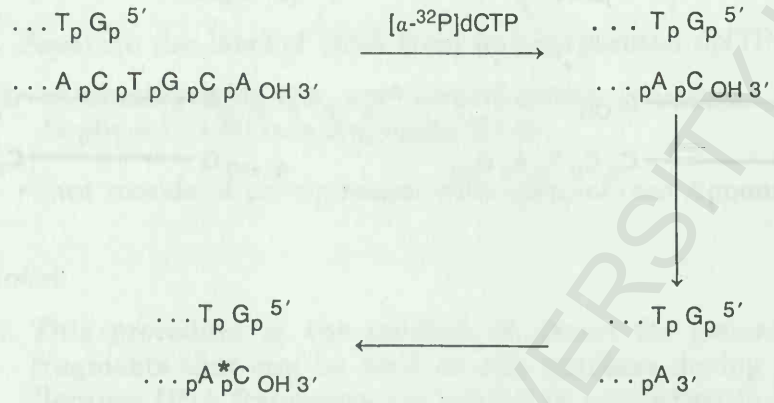
LABELING THE 3′ TERMINI OF DOUBLE-STRANDED DNA WITH BACTERIOPHAGE T4 DNA POLYMERASE

Rapid End-labeling of DNA

Bacteriophage T4 DNA polymerase, like the Klenow fragment of *E. coli* DNA polymerase I, can be used to label flush or recessed 3′ termini of DNA. However, bacteriophage T4 DNA polymerase carries a much more powerful 3′→5′ exonuclease activity than the Klenow fragment of *E. coli* DNA polymerase I, and it is therefore the enzyme of choice for end-labeling DNA molecules with protruding 3′ tails.



In many cases, a single buffer (given below) can be used both for the cleavage of DNA with a restriction enzyme and for the subsequent end-labeling. However, not all restriction enzymes work in bacteriophage T4 DNA polymerase buffer, and it is advisable to carry out pilot reactions with the particular batch of enzyme on hand. If the restriction enzyme will not work in the bacteriophage T4 DNA polymerase buffer, it is necessary to carry out the restriction endonuclease digestion and end-labeling in two separate steps. In this case, cleave the DNA in the appropriate restriction enzyme buffer, remove the restriction enzyme by extraction with phenol:chloroform, precipitate the DNA with ethanol, redissolve it in TE, and add the appropriate volume of 10× bacteriophage T4 DNA polymerase buffer.

1. Mix:

DNA	0.1–2 μg
10× bacteriophage T4 DNA polymerase buffer	2 μl
H_2O to 19 μl	

10× Bacteriophage T4 DNA polymerase buffer

0.33 M Tris-acetate (pH 8.0)
0.66 M potassium acetate
0.1 M magnesium acetate
5 mM dithiothreitol
1 mg/ml bovine serum albumin (Fraction V; Sigma)

The 10× stock should be divided into small aliquots and stored frozen at −20°C.

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2. Add the desired restriction enzyme and incubate for the appropriate time.

   If the desired restriction enzyme does not function in bacteriophage T4 DNA polymerase buffer, see discussion on page 10.54.

3. Add 1 μl of a solution containing three of the four dNTPs, each at a concentration of 2 mM.

4. Add 2–10 μCi (sp. act. 400–3000 Ci/mmole) of an aqueous solution of the fourth dNTP, labeled with α-^{32}P.

5. Add 1 μl (2.5 units) of bacteriophage T4 DNA polymerase.

6. Incubate the reaction for 5 minutes at 37°C.

7. Add 1 μl of a 2 mM solution of the unlabeled fourth dNTP. Continue the incubation for a further 10 minutes.

8. Stop the reaction by heating it to 70°C for 5 minutes.

9. Separate the labeled DNA from unincorporated dNTPs by

   - chromatography on, or centrifugation through, small columns of Sephadex G-50 (see Appendix E) or
   - two rounds of precipitation with ethanol (see Appendix E).

Note

In the presence of all four dNTPs, the $3' \rightarrow 5'$ exonuclease activity of the bacteriophage T4 DNA polymerase on double-stranded DNA is masked by the polymerization reaction. Thus, the product of the protocol described above will be a blunt-ended molecule with labeled dNTPs incorporated at or very near the termini of the DNA. It is possible to obtain more extensive labeling by a replacement reaction (see page 10.56).

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Replacement Synthesis

In the following reaction, the 3′→5′ exonuclease activity of bacteriophage T4 DNA polymerase first digests double-stranded DNA to produce molecules with recessed 3′ termini. On subsequent addition of radiolabeled dNTPs, the partially digested DNA molecules serve as primer:templates that are regenerated by the bacteriophage T4 DNA polymerase into intact double-stranded DNA.

Molecules labeled to high specific activity by this technique are used chiefly as hybridization probes. They have two advantages over probes prepared by nick translation. First, they lack the artifactual hairpin structures that can be produced during nick translation. Second, they can easily be converted into strand-specific probes by cleavage with suitable restriction enzymes (see Figure 10.6, page 10.58). In contrast to nick translation, however, this method does not produce a uniform distribution of radiolabel along the length of the DNA. Furthermore, the 3′→5′ exonuclease activity degrades single-stranded DNA much faster than double-stranded DNA, so that after a molecule has been digested to its midpoint, it will dissociate into two half-length single strands that will be rapidly degraded. It is therefore important to stop the exonuclease reaction before the enzyme reaches the center of the molecule. This is not always an easy task. The rate of exonucleolytic digestion is affected by the sequence of the DNA, and, for statistical reasons, different molecules in the population will be digested to different extents. Consequently, the replacement synthesis method yields a population of molecules that are fully labeled at their ends but that contain progressively decreasing quantities of label toward their centers.

1. Mix:

DNA	0.2–0.5 μg
10× bacteriophage T4 DNA polymerase buffer (see page 10.54)	2 μl
H_2O to 20 μl	

2. Add the desired restriction enzyme and incubate under the appropriate conditions.

   If the desired restriction enzyme does not function in bacteriophage T4 DNA polymerase buffer, see discussion on page 10.54.

3. Add bacteriophage T4 DNA polymerase. The amount of enzyme added and the ratio of enzyme to DNA in the reaction determine the rate of exonuclease digestion.

Ratio (units enzyme:μg DNA)	Average number of nucleotides removed from each 3′ terminus/minute
0.62	10
1.25	20
1.75	30
2.50	40

The rate of exonuclease digestion no longer increases linearly as a function of the enzyme:DNA ratio when the ratio exceeds 2.5.

4. Calculate the moles of nucleotide excised using the following equation:

$$M = \frac{D \times N}{B}$$

where M = moles of nucleotide excised, D = amount of DNA in the reaction (expressed as moles of nucleotide), N = number of nucleotides excised from one end of DNA, and B = number of nucleotide pairs in the DNA.

1 mole of nucleotide in DNA = 330 g.

5. Add 1 μl of a solution containing three of the four dNTPs, each at a concentration of 2 mM.

6. Add the fourth dNTP labeled with α-^{32}P (sp. act. 400–3000 Ci/mmole) in an amount at least equivalent to the moles of nucleotide excised from the DNA by the exonuclease. Incubate the reaction for 1 hour at 37°C.

7. Add 1 μl of a 2 mM solution of the fourth dNTP. Incubate the reaction for a further 15 minutes at 37°C.

8. Stop the reaction by heating it for 5 minutes at 70°C.

9. Separate the labeled DNA from unincorporated dNTPs by

   - chromatography on, or centrifugation through, small columns of Sephadex G-50 (see Appendix E) or
   - two rounds of precipitation with ethanol (see Appendix E).

Note

The specific activity of the replaced sequences will be about 7×10^8 cpm/μg if a single [α-^{32}P]dNTP with a specific activity of 400 Ci/mmole is used.

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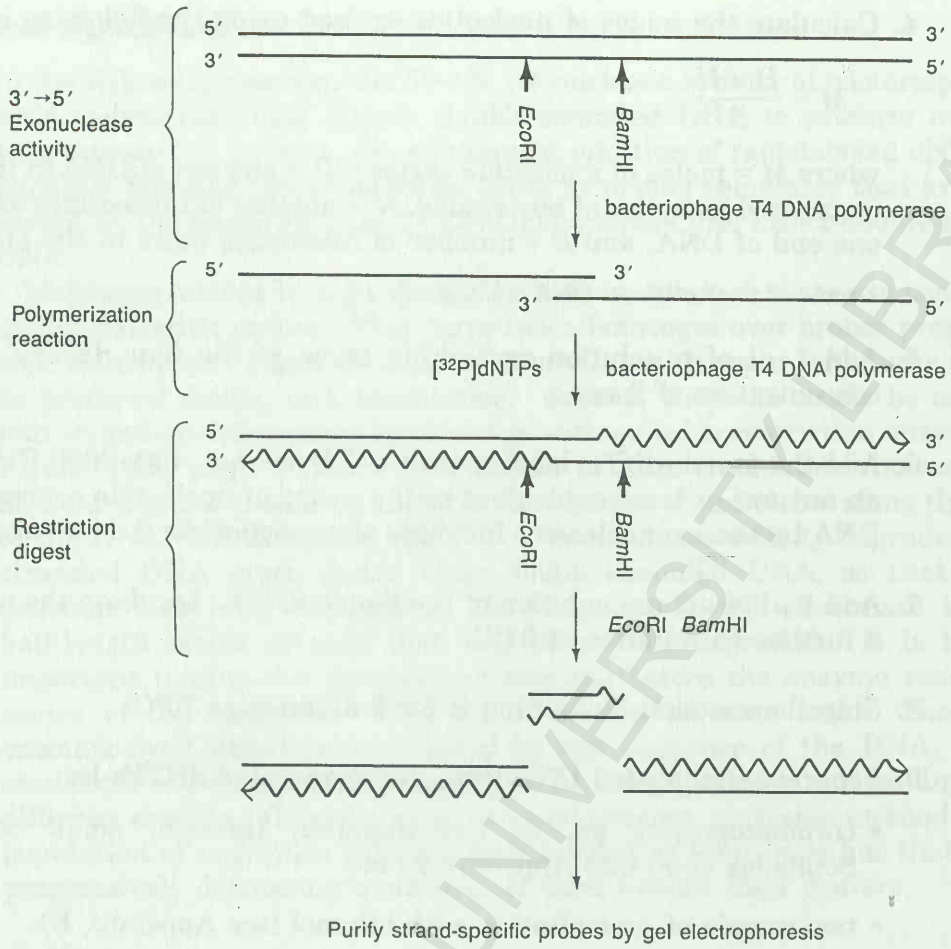

FIGURE 10.6
Production of strand-specific hybridization probes.

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LABELING THE 5′ TERMINUS OF DNA WITH BACTERIOPHAGE T4 POLYNUCLEOTIDE KINASE

The transfer of ^{32}P to the 5′ terminus of DNA can be catalyzed by bacteriophage T4 polynucleotide kinase in two ways (Figure 10.7). In the forward reaction, the γ-^{32}P of [γ-^{32}P]ATP is transferred to a hydroxyl group created by the removal of phosphate from the 5′ terminus of DNA by alkaline phosphatase. In the exchange reaction, the unlabeled phosphate is transferred from DNA to ADP, and the dephosphorylated DNA is then rephosphorylated with ^{32}P.

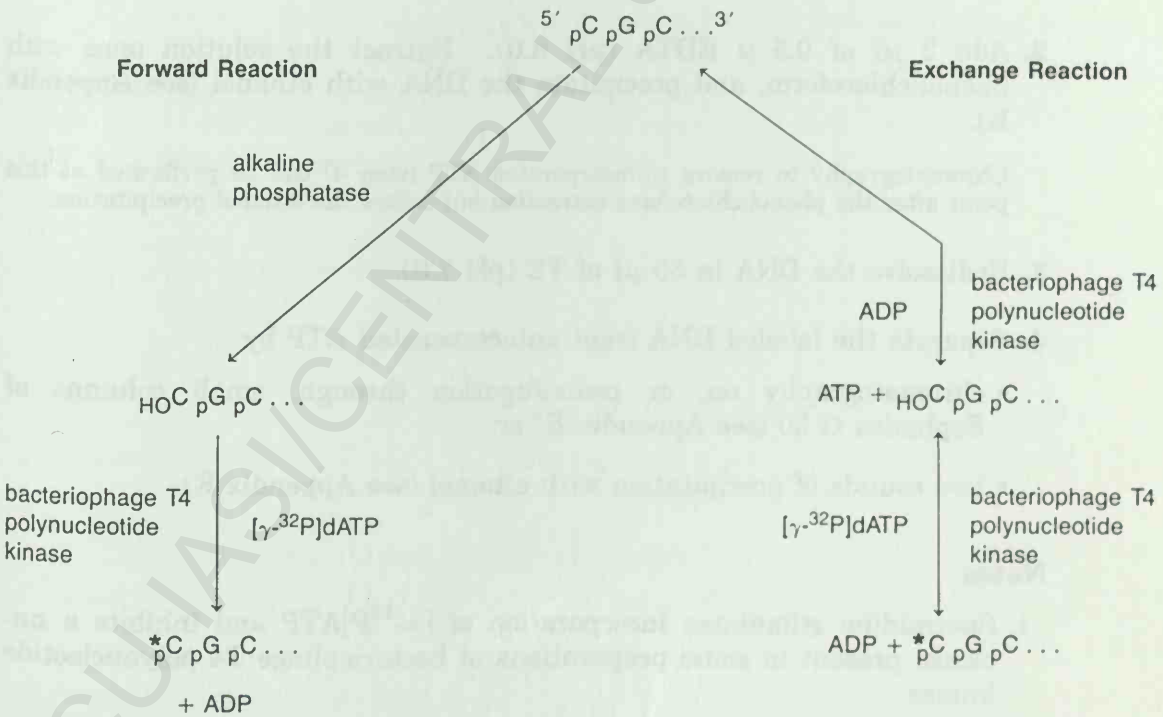

FIGURE 10.7
Forward and exchange reactions of bacteriophage T4 DNA polymerase.

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Forward Reaction

USING DNA MOLECULES WITH PROTRUDING 5′ TERMINI AS SUBSTRATES

1. Mix:

5′ termini of dephosphorylated DNA (see pages 10.64–10.65)	1–50 pmoles
10 × bacteriophage T4 polynucleotide kinase buffer	5 μl
[γ-^{32}P]ATP (sp. act. 3000 Ci/mmole; 10 μCi/μl)	50 pmoles
bacteriophage T4 polynucleotide kinase	10–20 units
H_2O to 50 μl	

Incubate the reaction for 30 minutes at 37°C.

1 mole of 5′ termini = 0.5 mole of double-stranded DNA. For example:
1 mole of linear pBR322 DNA = 3.2×10^6 g
1 mole of 5′ termini of linear pBR322 DNA = 1.6×10^6 g
1 pmole of 5′ termini of linear pBR322 DNA = 1.6 μg

10 × Bacteriophage T4 polynucleotide kinase buffer

0.5 M Tris · Cl (pH 7.6)
0.1 M $MgCl_2$
50 mM dithiothreitol
1 mM spermidine HCl
1 mM EDTA (pH 8.0)

2. Add 2 μl of 0.5 M EDTA (pH 8.0). Extract the solution once with phenol:chloroform, and precipitate the DNA with ethanol (see Appendix E).

Chromatography to remove unincorporated ATP (step 4) can be performed at this point after the phenol:chloroform extraction but before the ethanol precipitation.

3. Redissolve the DNA in 50 μl of TE (pH 7.6).

4. Separate the labeled DNA from unincorporated ATP by

- chromatography on, or centrifugation through, small columns of Sephadex G-50 (see Appendix E) or
- two rounds of precipitation with ethanol (see Appendix E).

Notes

i. Spermidine stimulates incorporation of [γ-^{32}P]ATP and inhibits a nuclease present in some preparations of bacteriophage T4 polynucleotide kinase.

ii. The concentration of ATP in the reaction should be at least 1 μM.

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iii. The dephosphorylated DNA should be rigorously purified by gel electrophoresis, density gradient centrifugation, or chromatography on columns of Sepharose CL-4B to free it from low-molecular-weight nucleic acids. Although such contaminants may make up only a small fraction of the weight of the nucleic acid in the preparation, they contribute a much larger proportion of the 5′ termini. Unless steps are taken to remove them, contaminating low-molecular-weight DNAs and RNAs can be the predominant species of nucleic acids that are labeled in bacteriophage T4 polynucleotide kinase reactions.

iv. Ammonium ions are strong inhibitors of bacteriophage T4 polynucleotide kinase; therefore, DNA should not be dissolved in, or precipitated from, buffers containing ammonium salts prior to treatment with kinase.

v. The efficiency of incorporation of γ-^{32}P into oligonucleotides can be measured by thin-layer chromatography on polyethyleneimine plates (see Chapter 11, page 11.32).

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USING DNA MOLECULES WITH BLUNT ENDS OR RECESSED 5′ TERMINI AS SUBSTRATES

DNA molecules with blunt ends or recessed 5′ termini are labeled less efficiently with bacteriophage T4 polynucleotide kinase than molecules with protruding 5′ termini. Even a partial reaction at recessed 5′ termini requires large amounts of enzyme and ATP. However, the efficiency of labeling may be greatly improved by including polyethylene glycol in the reaction (Harrison and Zimmerman 1986).

1. Mix in the order given:

5′ termini of dephosphorylated DNA in a volume of 11 μl or less	1–50 pmoles
10× imidazole buffer	4 μl
H_2O to 15 μl	
polyethylene glycol (PEG 8000) (24% w/v)	10 μl

10× Imidazole buffer

500 mM imidazole · Cl (pH 6.4)
180 mM $MgCl_2$
50 mM dithiothreitol

2. Add at least 50 pmoles of [γ-^{32}P]ATP (sp. act. 3000 Ci/mmole; 10 μCi/μl) in a volume of 15 μl.

3. Add 20 units of bacteriophage T4 polynucleotide kinase. Mix, and incubate the reaction for 30 minutes at 37°C.

4. Add 1 μl of 0.5 M EDTA (pH 8.0). Extract the mixture once with chloroform, and precipitate the DNA with 2 volumes of ice-cold ethanol. Store the tube on ice for 15 minutes, and then collect the precipitated DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge.

   Chromatography to remove unincorporated ATP (step 6) can be performed at this point after the extraction with chloroform but before the ethanol precipitation.

5. Redissolve the DNA in 50 μl of TE (pH 7.6).

6. Separate the labeled DNA from unincorporated ATP by

   - chromatography on, or centrifugation through, small columns of Sephadex G-50 (see Appendix E) or
   - two rounds of precipitation with ethanol (see Appendix E).

Notes

i. In the presence of polyethylene glycol and magnesium, DNA undergoes a "psi" transition and collapses into a highly condensed state (Lerman 1971). The increased efficiency of the phosphorylation reaction is thought

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to be a consequence of the resulting macromolecular crowding (Harrison and Zimmerman 1986). The amount of stimulation is critically dependent on the concentration of polyethylene glycol. It may therefore be useful to test the efficiency of the reaction in concentrations of polyethylene glycol ranging between 4% and 10%.

ii. The beneficial effects of polyethylene glycol only become apparent when using DNAs longer than 300 bp. Smaller fragments of DNA probably cannot undergo the "psi" transition.

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DEPHOSPHORYLATION OF DNA

Terminal 5′ phosphates can be removed from DNA (or RNA) by treatment with alkaline phosphatase. For several years, bacterial alkaline phosphatase was used for this purpose, but highly purified calf intestinal alkaline phosphatase (CIP) has recently become available from commercial suppliers, and it is now the enzyme of choice. CIP has the considerable advantage that it can be completely inactivated by heating to 65°C in SDS and EDTA.

1. Digest the DNA to completion with the restriction enzyme of choice. Purify the DNA by extraction with phenol:chloroform and precipitation with 2 volumes of ice-cold ethanol for 15 minutes at 0°C. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge.

2. Dissolve the DNA in a minimum volume of 10 mM Tris · Cl (pH 8.3).

3. Add:

   10 × CIP dephosphorylation buffer 5 μl
   H_2O to 48 μl

   10 × CIP dephosphorylation buffer

   10 mM $ZnCl_2$
   10 mM $MgCl_2$
   100 mM Tris · Cl (pH 8.3)

   Add 0.01 unit of CIP. This is sufficient to remove the terminal phosphates from 1 pmole of protruding 5′ termini of DNA (1 pmole of termini of a 4.4-kb linear DNA is 1.6 μg); 0.1–0.5 unit of CIP is needed to remove the terminal phosphates from 1 pmole of blunt ends or recessed 5′ termini of DNA.

   To dephosphorylate protruding 5′ termini, incubate for 30 minutes at 37°C; add a second aliquot of CIP, and continue incubation for a further 30 minutes. To dephosphorylate DNA with blunt ends or recessed 5′ termini, incubate for 15 minutes at 37°C; add another aliquot of CIP, and continue incubation for a further 45 minutes at 55°C.

4. At the end of the incubation period, add SDS and EDTA (pH 8.0) to final concentrations of 0.5% and 5 mM, respectively. Mix well, and add proteinase K to a final concentration of 100 μg/ml. Incubate for 30 minutes at 56°C.

   Proteinase K is used to digest the CIP, which must be completely removed if subsequent ligations are to work efficiently. An alternative method is to inactivate the CIP by heating to 65°C for 1 hour (or 75°C for 10 minutes) in the presence of 5 mM EDTA (pH 8.0) and then to purify the dephosphorylated DNA by extraction with phenol:chloroform.

5. Cool the reaction to room temperature, and purify the DNA by extracting once with phenol:chloroform and once with chloroform alone.

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6. Recover the aqueous phase, and add 0.1 volume of 3 M sodium acetate (pH 7.0). Mix well, and add 2 volumes of ethanol at 0°C. Mix well, and store at 0°C for at least 2 hours. Collect the precipitate by centrifugation at 12,000*g* for 20 minutes at 4°C in a microfuge. Wash the pellet with ice-cold 70% ethanol and recentrifuge. Allow the precipitate to dry completely at room temperature before dissolving it in TE (pH 7.6) at a concentration of more than 2 nmoles/ml.

Notes

i. The pH of the 3 M sodium acetate solution is 7.0 rather than the usual 5.2. At acid pH, EDTA precipitates from solution if its concentration exceeds 5–10 mM.

ii. Until recently, CIP was supplied as a suspension in a slurry of ammonium sulfate. However, it may now be obtained as a stabilized solution (in 30 mM triethanolamine buffer [pH 7.6], 3 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$). It should be stored undiluted in this buffer at 4°C (not frozen).

iii. To remove 5′ phosphates from RNA, use 0.01 unit of CIP per picomole of 5′ termini. Incubate for 15 minutes at 37°C, followed by 30 minutes at 55°C.

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USING DNA MOLECULES WITH PROTRUDING 5′-PHOSPHATE TERMINI AS TEMPLATES

This procedure (Berkner and Folk 1977) is faster than the forward reaction because it does not require that the DNA be dephosphorylated. The efficiency of the reaction is greatly improved by including polyethylene glycol in the reaction (Harrison and Zimmerman 1986).

1. Mix in the following order:

5′ termini of DNA with 5′-terminal phosphates	1–50 pmoles
10 × exchange-reaction buffer	5 μl
1 mM ADP	5 μl
50 nM ATP	1 μl
[γ-^{32}P]ATP (sp. act. 3000 Ci/mmole; 10 μCi/μl)	50 pmoles
H_2O to 40 μl	
polyethylene glycol (PEG 8000) (24% w/v)	10 μl
bacteriophage T4 polynucleotide kinase (20 units)	1 μl

10 × Exchange-reaction buffer

500 mM imidazole · Cl (pH 6.4)
180 mM $MgCl_2$
50 mM dithiothreitol
1 mM spermidine HCl
1 mM EDTA (pH 8.0)

2. Incubate the reaction for 30 minutes at 37°C.

3. Add 2 μl of 0.5 M EDTA (pH 8.0).

4. Extract once with chloroform and precipitate the DNA with 2 volumes of ice-cold ethanol for 15 minutes at 0°C. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge.

   Chromatography to remove unincorporated ATP (step 6) can be performed at this point after the extraction with chloroform but before the ethanol precipitation.

5. Redissolve the DNA in 50 μl of TE (pH 7.6).

6. Separate the labeled DNA from unincorporated ATP by

   - chromatography on, or centrifugation through, small columns of Sephadex G-50 (see Appendix E) or
   - two rounds of precipitation with ethanol (see Appendix E).

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Notes

i. In the presence of polyethylene glycol and magnesium, DNA undergoes a "psi" transition and collapses into a highly condensed state (Lerman 1971). The increased efficiency of the phosphorylation reaction is thought to be a consequence of the resulting macromolecular crowding (Harrison and Zimmerman 1986). The amount of stimulation is critically dependent on the concentration of polyethylene glycol. It may therefore be useful to test the efficiency of the reaction in concentrations of polyethylene glycol ranging between 4% and 10%.

ii. The beneficial effects of polyethylene glycol only become apparent when using DNAs longer than 300 bp. Smaller fragments of DNA probably cannot undergo the "psi" transition.

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11
Synthetic Oligonucleotide Probes

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Methods to synthesize short tracts of single-stranded DNA, first developed over 20 years ago, have now been refined to the point where almost all chemical synthesis of oligonucleotides is carried out by highly automated machines. The consequent ready availability of oligonucleotides of defined sequence, coupled with the development of appropriate hybridization techniques, has revolutionized molecular cloning in two ways. First, it has led to the primary isolation of cloned copies of large numbers of genes that were previously inaccessible. Today, knowledge of the sequence of a few amino acids in a protein is sufficient to allow the rapid isolation of cloned copies of the corresponding gene. Second, it has led to the development of methods to introduce mutations at specific sites in cloned copies of genes whose sequences are known. It is therefore possible to delete and insert segments of DNA and generate complete canons of point mutations at chosen sites in the genes of interest. In this chapter, we

- Describe the types of oligonucleotide probes and their uses
- Present methods for the labeling and purification of oligonucleotide probes
- Present general guidelines to allow selection of conditions that promote maximal specificity of hybridization

The chemical synthesis of oligonucleotides will not be discussed, and the reader is referred to articles by Narang (1983) and Itakura et al. (1984) for descriptions of the chemistry used in currently available automated synthesizers. The use of oligonucleotides in site-directed mutagenesis is described in detail in Chapter 15.

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Types and Uses of Oligonucleotide Probes

Three types of oligonucleotide probes are in common use:

- Single oligonucleotides of defined sequence
- Pools of short oligonucleotides whose sequences are highly degenerate
- Pools of longer oligonucleotides of lesser degeneracy

The *degeneracy* of oligonucleotide pools that are used to screen libraries by hybridization is a consequence of the degeneracy of the genetic code itself. There are 64 possible codons and only 20 amino acids; most amino acids are represented by more than one codon. Thus, a certain sequence of amino acids can be encoded by a number of different oligonucleotides. A completely degenerate pool of oligonucleotides contains all DNA sequences that can code for a given sequence of amino acids. A pool of limited degeneracy consists of a selected subset of oligonucleotides that can code for a given sequence of amino acids.

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SINGLE OLIGONUCLEOTIDES OF DEFINED SEQUENCE

Probes consisting of a single oligonucleotide of defined sequence usually correspond to part of the sequence of a previously cloned segment of DNA. In general, such oligonucleotides match with their target sequence perfectly, or nearly perfectly, and they are sufficiently long (19–40 nucleotides) to allow the use of hybridization conditions that can guarantee discrimination between the target sequence and other closely related sequences. Single oligonucleotides of defined sequence are used:

- To screen cDNA or genomic DNA libraries, or subclones derived from them, for additional clones containing segments of DNA that have been isolated previously and sequenced
- In Southern, northern, or dot-blot hybridization to identify or detect the sequences of specific genes
- In Southern or dot-blot hybridization of genomic DNA to detect specific mutations in genes of known sequence
- To detect specific mutations generated by site-directed mutagenesis of cloned genes
- To map the 5′ termini of mRNA molecules by primer extension

Oligonucleotide probes used for these purposes are usually labeled by phosphorylation of 5′ termini with [γ-^{32}P]ATP (see pages 11.31–11.32) or, more rarely, by synthesis of a radioactive complementary strand with the Klenow fragment of *E. coli* DNA polymerase I (see pages 11.40–11.44). Because the base composition and size of each oligonucleotide probe are known precisely, conditions can be defined that allow hybridization of the oligonucleotide to its desired target and little or no hybridization to closely related sequences that are not perfectly matched (see below). Obviously, such probes are limited to applications where the sequence of the target DNA or RNA is already known (see, e.g., Montgomery et al. 1978); they are of no use when screening libraries to isolate clones of unknown sequence.

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POOLS OF SHORT OLIGONUCLEOTIDES WHOSE SEQUENCES ARE HIGHLY DEGENERATE

Degenerate pools of short oligonucleotides containing all possible sequences that can code for a given tract of amino acids have been used as hybridization probes to isolate both cDNA clones (see, e.g., Goeddel et al. 1980; Sood et al. 1981; Suggs et al. 1981a; Wallace et al. 1981) and genomic DNA clones (see, e.g., Toole et al. 1984; Jacobs et al. 1985; Lin et al. 1985). The sequences of these oligonucleotides are typically deduced, using the genetic code, from short tracts of amino acid sequence determined by the sequencing of small quantities of highly purified proteins (see, e.g., Hewick et al. 1981; Hunkapiller et al. 1983; Kent et al. 1987). Because of the degeneracy of the genetic code, several oligonucleotides must usually be synthesized to represent all possible ways to code for a given short sequence of amino acids. For example:

Amino acid sequence:	VAL	ASN	PHE	TYR	ALA	TRP	LYS
DNA coding sequences:	GT A T C G	AA T C	TT T C	TA T C	GC A G C	TGG	AA A G

The sequence within the shaded region contains three codons with twofold ambiguity and one codon with a fourfold ambiguity. Thus, a comprehensive pool containing $2 \times 2 \times 2 \times 4 = 32$ related oligonucleotides, each 17 nucleotides in length, would contain all possible ways to code for the amino acid sequence Asn Phe Tyr Ala Trp Ly:

AATTTTTATGCATGGAA
AACTTTTATGCATGGAA
AATTTCTATGCATGGAA
AACTTCTATGCATGGAA
AATTTTTACCGATGGAA
etc.

The presence of all potential coding combinations in this pool ensures that one of the 32 oligonucleotides will be an exact match for the corresponding genomic DNA or cDNA sequence. Thus, if hybridization conditions can be found under which only perfectly matched sequences form stable duplexes, cloned copies of the gene of interest can be readily isolated from cDNA or genomic DNA libraries. However, it is important to realize that not all strongly hybridizing clones will necessarily contain sequences of the gene of interest. There may be other oligonucleotides in the pool that by chance match perfectly with the sequences of other cloned genes. Furthermore, hybridization conditions cannot always be established that allow only perfectly matched hybrids to form, which leads to the isolation of other clones containing sequences that are closely related, but not identical, to any one of the 32 oligonucleotides in the pool. In practice, these problems are not serious.

Pools of degenerate oligonucleotides are used, not to identify target clones unambiguously, but to select a series of candidate clones that can then be tested further. To ensure that the target clone is represented in the initial group of candidates, screening conditions are usually chosen that allow

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detection of perfect hybrids formed between the most A/T-rich oligonucleotide in the pool and its potential target. Other hybrids, both perfect and imperfect, that are stable under these conditions will identify additional candidate clones. The total number of positive clones, which is normally not large when screening cDNA libraries, can be reduced either by designing longer oligonucleotides (or by decreasing their degeneracy) or by using hybridization solvents, such as tetramethylammonium chloride (Jacobs et al. 1985; Wood et al. 1985), in which the stability of hybrids is dependent only on length and not on content of G + C. In addition, the group of candidates identified by one pool of oligonucleotides can be screened by a second oligonucleotide pool derived from a separate tract of amino acid sequence. Clones that hybridize to two independent pools of oligonucleotides usually contain the desired target gene or cDNA.

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The Effects of Length and Degeneracy of the Oligonucleotide on the Specificity of Hybridization

The number of independent perfect matches (P_o) expected for an oligonucleotide of length L in a genome of complexity C can be calculated from the equation:

$$P_o = (1/4)^L \cdot 2C$$

The complexity, which is measured in nucleotide pairs, is the sum of the nucleotide pairs that occur in unique (single-copy) sequences and those that occur in one copy of each repeated sequence. Thus, nucleotide pairs belonging to repetitive elements, which may occur many times in a genome, are counted only once. Only approximate values of C are known for genomes that have not been sequenced completely. The following estimates are taken from Laird (1971).

Genome	Size[a]	Complexity[a]
Escherichia coli	4.0×10^6	$\sim 4.0 \times 10^6$
Yeast	1.4×10^7	$\sim 1.4 \times 10^7$
Drosophila	1.1×10^8	$\sim 1.05 \times 10^8$
Mammals	2.9×10^9	$\sim 1.8 \times 10^9$

[a]Numbers are given in nucleotide pairs.

The term $2C$, rather than C, is used in the equation because the sequence of each strand of DNA is different and has the potential to hybridize to the probe. As the length of an oligonucleotide increases, the chance that it will find an exact match in the genome of interest decreases. When $4^L = 2C$, the sequence of the oligonucleotide would be expected to occur only once in the genome. In the case of mammalian genomes, $2C \cong 3.6 \times 10^9$, and an oligonucleotide of 14–15 nucleotides would therefore be expected to be represented only once in the genome. However, the distribution of nucleotides in the coding sequences of mammalian genomes is nonrandom (Lathe 1985), and it is therefore advisable to use longer oligonucleotides in order to increase the specificity of hybridization. For example, when the oligonucleotide is 16 nucleotides long, there is only one chance in ten that a typical mammalian cDNA library ($C \cong 10^7$ nucleotides) will fortuitously contain a sequence that exactly matches the sequence of the oligonucleotide. Thus, any clones that hybridize strongly to the probe are likely to be derived from the gene of interest. Bear in mind that when a cDNA library is screened with an oligonucleotide probe, there is no relationship between the observed number of positive clones and their frequency predicted by statistics. For example, if by chance the oligonucleotide should match a sequence that is abundantly represented in mRNA, the number of clones that hybridize to the probe will be much larger than theory predicts.

The effect of hybridization between imperfectly matched sequences cannot be easily quantitated, since different types of mismatch (mispairing between single bases, loopouts on either strand, multiple mismatches closely or distantly spaced) have different effects on the stability of double-stranded DNA. For example, a single mismatch in the center of a short oligonucleotide (14–16 nucleotides in length) will eliminate any possibility of hybridization

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under any practicable conditions. However, a mismatch at the end of such an oligonucleotide, or in the center of a longer oligonucleotide, may have no detectable effect (Ikuta et al. 1987).

Assuming that all mismatches at all positions are equivalent, the number of clones (P_K) that will hybridize detectably to an oligonucleotide of length L with K mismatches is given by the equation:

$$P_K = (1/4)^{L-K} \cdot (3/4)^K \cdot 2C \cdot L!/[K! \cdot (L-K)!]$$

For the simplest case (that of a single mismatch, where $K = 1$):

$$P_1 = (1/4)^{L-1} \cdot (3/4) \cdot 2C \cdot L$$

Compare this with the number of perfect matches:

$$P_0 = (1/4)^L \cdot 2C$$

The ratio $P_1/P_0 = 3L$. Thus, if the hybridization conditions allow detection of singly mismatched sequences, a 24-mer will hybridize to 72-fold more mismatched clones than perfectly matched clones.

When the oligonucleotide is close to the critical length (which for screening of a typical mammalian cDNA library is 17–18 nucleotides [Lathe 1985]), it is worthwhile increasing its length in order to maximize its specificity. The concomitant increase in mismatches that occurs as the degeneracy of the pool increases is not a significant problem when dealing with such small oligonucleotides, since mispaired sequences are unlikely to be stable under practicable conditions of hybridization. Since the decrease in the stability of a hybrid is approximately 1–1.5°C for every percentage of mismatch, the melting temperature of a hybrid 20 nucleotides in length would be reduced 5–7.5°C by internal mismatches. This is sufficient leeway to allow easy discrimination between perfectly matched and internally mismatched hybrids formed by short (<20 nucleotides) oligonucleotides. However, as the length of the oligonucleotide increases beyond 20 nucleotides, the effect of mismatches on the melting temperature of the hybrid becomes progressively less significant, and it becomes difficult to establish hybridization conditions that will discriminate between perfectly matched and internally mismatched hybrids. When the oligonucleotide is more than 20 nucleotides long, many mismatches are likely to be stable under the conventional conditions used for hybridization. This can result in the isolation of an unacceptably high number of candidate clones, many of which will turn out not to contain the gene of interest. In summary, oligonucleotides in degenerate pools should be long enough to hybridize specifically to the gene of interest and short enough not to form internally mismatched hybrids that can be detected by hybridization. For example, screening of mammalian cDNA or genomic DNA libraries should be carried out with degenerate pools of oligonucleotides 17–20 nucleotides in length. When longer probes are used, it is usually necessary to take steps to limit the degeneracy of the pool as much as possible (see pages 11.11–11.19).

Designing Degenerate Pools of Short Oligonucleotides

The number and length of oligonucleotides that are used to screen cDNA and genomic DNA libraries are usually determined by the amount of amino acid sequence information that is available. The following guidelines should be used to design degenerate pools that correspond to each separate tract of amino acids.

1. The entire available protein sequence should be examined for regions rich in amino acids that are specified by only one or two codons. Regions rich in amino acids with six codon choices (Leu, Ser, and Arg) should be avoided. The number of codons that encode each amino acid are given below (see also Figure 11.1).

1	2	3	4	6
Met	Phe	Ile	Val	Leu
Trp	Tyr		Pro	Ser
	His		Thr	Arg
	Gln		Ala	
	Asn		Gly	
	Lys			
	Asp			
	Glu			
	Cys			

2nd position of codon

1st position of codon (5′ terminus)	U		C		A		G		3rd position of codon (3′ terminus)
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys	C
	UUA	Leu	UCA	Ser	UAA	Stop (Ochre)	UGA	Stop (Umber)	A
	UUG	Leu	UCG	Ser	UAG	Stop (Amber)	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC	Leu	CCC	Pro	CAC	His	CGC	Arg	C
	CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	A
	CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg	G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser	C
	AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	A
	AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg	G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly	C
	GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly	A
	GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly	G

FIGURE 11.1
The genetic code (nuclear genes).

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2. If an amino acid with six possible codons must be used, synthesize two separate pools of oligonucleotides so that only the six correct codons are represented. In the case of Leu, for example, divide the synthesis into two pools, one containing CTA, CTG, CTT, and CTC and the other containing TTA and TTG. In this way, it is possible to avoid incorporating the two incorrect codons TTT and TTC (both of which code for Phe) into the oligonucleotide.

3. If enough amino acid sequence is available, it may be possible to design nonoverlapping or partially overlapping probes that are complementary to different regions of the coding sequence. By selecting only those clones that hybridize to both probes, the number of recombinants detected as a consequence of mismatches is greatly reduced. The second probe can be considerably shorter or more degenerate than the first, since the vast bulk of the library has been reduced to the relatively small number of clones that hybridize to the first probe.

4. Use the most stringent hybridization conditions possible. This aspect of the use of oligonucleotide probes is discussed in detail on page 11.45. Typically, degenerate pools contain oligonucleotides of different base composition, which will form hybrids that melt at quite different temperatures. However, solvents are now available in which the melting temperature of the hybrid is largely independent of base composition (see pages 11.48–11.49).

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POOLS OF LONGER OLIGONUCLEOTIDES OF LESSER DEGENERACY

Two methods are used to increase the length of oligonucleotide probes while limiting the degeneracy of the pools used for hybridization:

- Hybridization with oligonucleotides that contain only a subset of the possible codons at each position ("guessmers"). In many cases, only a single oligonucleotide 30–70 nucleotides in length is synthesized that contains the combination of codons most likely to match the authentic gene.
- Hybridization with oligonucleotides that contain a "neutral" base, such as inosine, at each position of ambiguity. By pairing in an equivalent fashion with all four conventional bases, inosine neither confers stability on double-stranded helices nor detracts from it (Martin et al. 1985).

These methods work because the detrimental effects of mismatches or neutral bases are outweighed by the increased stability of hybrids formed by longer oligonucleotides. Most amino acids are specified by codons that differ only in the third position, so that at least two of the three nucleotides of each codon are guaranteed to be perfectly matched. Hybridization can therefore be carried out under comparatively stringent conditions. However, these methods suffer from a major limitation: They can only be used when a sufficiently long stretch of amino acid sequence is available.

Guessmers

Longer oligonucleotides of limited degeneracy were first used by Jaye et al. (1983) to isolate a cDNA clone coding for human factor IX and by Anderson and Kingston (1983) to isolate a genomic clone of bovine pancreatic trypsin inhibitor. Subsequently, cDNA and genomic DNA clones of many other genes have been obtained in a similar fashion (Table 11.1), and the use of guessmers is now the method of choice for the primary isolation of clones when sufficient amino acid sequence is available.

There are two problems in using a guessmer: (1) designing the oligonucleotide so that its sequence has the highest probability of matching the gene of interest and (2) finding conditions that promote hybridization of the probe to the target sequence and limit its annealing to irrelevant sequences.

DESIGNING A GUESSMER

There are no rules that guarantee selection of the correct codon at a position of ambiguity; neither is it possible to predict how many correct choices must be made to achieve success. On the basis of mathematical calculations (Lathe 1985), a probe would be expected to have at least 76% homology with the authentic gene even if all codon choices were made on a random basis. If substitutions are chosen on the statistical basis of known codon utilization in the species of interest, the expected homology increases to 82%; it rises still further (to 86%) if regions lacking leucine, arginine, and serine are chosen (each of these three amino acids is specified by six codons). Clearly, as the degree of homology increases, the guessmer becomes a more specific probe for

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TABLE 11.1 Conditions for Screening Mammalian cDNA or Genomic DNA Libraries with Guessmers

Oligonucleotide		Hybridization conditions		Wash conditions		Percent mismatch	Gene	Reference
length	percent G + C							
86	63	0.67 M Na^+	65°C	0.67 M Na^+	65°C	19	bovine pancreatic trypsin inhibitor	Anderson and Kingston (1983)
52	46	1.2 M Na^+	48°C	1.2 M Na^+	48°C	17.3	human coagulation factor IX	Jaye et al. (1983)
74	54	0.98 M Na^+ 20% formamide	42°C	0.04 M Na^+	40°C	24	human transforming growth factor-α	Derynck et al. (1984)
51	68	0.98 M Na^+ 20% formamide	42°C	0.04 M Na^+	45°C	10	human epidermal growth factor receptor	Ullrich et al. (1984b)
81	55	0.98 M Na^+ 20% formamide	42°C	0.04 M Na^+	40°C	20	human insulin-like growth factor I	Ullrich et al. (1984a)
63	63	0.98 M Na^+ 20% formamide	42°C	0.04 M Na^+	40°C	14	human insulin receptor	Ullrich et al. (1985)
54	77	0.98 M Na^+ 20% formamide	42°C	0.04 M Na^+	40°C	21	human insulin receptor	Ullrich et al. (1985)
42	54	0.98 M Na^+ 20% formamide	42°C	0.04 M Na^+	40°C	21	human tumor necrosis factor	Pennica et al. (1984)
45	50	0.98 M Na^+ 20% formamide	45°C	0.98 M Na^+	50°C	11	human coagulation factor VIII	Toole et al. (1984)
36	50	1.08 M Na^+ 20% formamide	42°C	0.195 M Na^+	37°C	17	human coagulation factor VIII	Wood et al. (1984)
44	50	0.98 M Na^+ 20% formamide	42°C	0.04 M Na^+	45°C	29	human transforming growth factor-β	Derynck et al. (1985)
64	64	0.98 M Na^+ 40% formamide	37°C	0.2 M Na^+	50°C	17	porcine inhibin α-subunit	Mason et al. (1985)

56	64	0.98 M Na^+ 40% formamide	37°C	0.2 M Na^+	50°C	12.5	porcine inhibin β-subunit	Mason et al. (1985)
69	63	0.98 M Na^+	42°C	0.1 M Na^+	50°C	16	human tissue inhibitor of metalloproteinases	Docherty et al. (1985)
48	60	0.98 M Na^+ 20% formamide	42°C	0.04 M Na^+	42°C	12	signal recognition particle receptor	Lauffer et al. (1985)
86	66	0.98 M Na^+ 20% formamide	42°C	0.1 M Na^+	42°C	22	human platelet-derived growth factor A-chain	Betsholtz et al. (1986)
32	53	0.98 M Na^+	45°C	0.98 M Na^+	50°C	12	human protein kinase C	Knopf et al. (1986)
59	54	1.2 M Na^+	38–40°C	0.2 M Na^+	37°C	27	bovine chromogranin A	Benedum et al. (1986)
48	64	1.2 M Na^+ 20% formamide	42°C	0.2 M Na^+	55°C	11–14	bovine basic fibroblast growth factor	Abraham et al. (1986)
78	53	0.98 M Na^+ 20% formamide	42°C	0.04 M Na^+	40°C	18	human insulin-like growth factor I receptor	Ullrich et al. (1986)
66	57	0.90 M Na^+	65°C	0.90 M Na^+	65°C	10	human factor XIIIa	Grundmann et al. (1986)
50[a]	52	0.98 M Na^+	45°C	0.98 M Na^+	45°C	16	rat bone Gla protein	Celeste et al. (1986)
35	77	0.98 M Na^+	45°C	0.98 M Na^+	45°C	11	rat bone Gla protein	Celeste et al. (1986)
32[b]	59	0.98 M Na^+	45°C	0.98 M Na^+	45°C	3	rat cartilage proteoglycan	A.J. Celeste et al. (unpubl.)
32	41	0.98 M Na^+	45°C	0.98 M Na^+	45°C	9	rat cartilage proteoglycan	A.J. Celeste et al. (unpubl.)
50	56	1.6 M Na^+ 20% formamide	42°C	60 mM Na^+	42°C	22	human G_{α_s} signal transduction protein	Bray et al. (1986)

This table contains a representative sample of the conditions used to screen mammalian cDNA or genomic DNA libraries with guessmers. It was not our aim to present a listing of all genes that have been isolated in this way.

[a]Eightfold degenerate.

[b]Twofold degenerate.

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the gene of interest and forms hybrids that are stable under a wider range of hybridization conditions. Comparison of the sequences of guessmers with those of the various genes they were used to isolate (see Table 11.1) shows that the amount of homology ranges from a low of 71% to as high as 97%. Unless your choice of codons is extremely unfortunate, you should be able to design guessmers whose homology with their target genes lies near the upper end of this range. However, in addition to overall homology, the presence of a perfectly matching sequence of contiguous nucleotides within the guessmer may also be important for success. Almost all guessmers that have been used successfully contain regions that match exactly with sequences in their target genes. Although it is not possible to guarantee that a guessmer will contain an exactly matching sequence, the chances increase dramatically as the degree of overall homology rises.

When designing a guessmer:

1. Eliminate codon choices that generate the sequence CpG between codons. This dinucleotide is significantly underrepresented in mammalian DNA (Bird 1980), and CpG occurs in intercodons at about one half of the expected frequency (Lathe 1985).

2. Choose the codon that most commonly codes for a particular amino acid in the species under study. Currently, the most comprehensive lists of relative codon frequencies in mammalian genes can be found in Grantham et al. (1980, 1981), Lathe (1985), and Sharp et al. (1988). Similar data for yeast genes have been compiled by Bennetzen and Hall (1982), Davis and Thorner (1987), and Hoekema et al. (1987). In addition, Sharp et al. (1988) have reviewed the considerable within-species diversity of codon usage. When a combination of the most commonly used codons generates an intercodon containing the dinucleotide CpG, replace one codon with the next most commonly used codon.

3. Remember that specific gene families occasionally display marked bias for or against certain codons (e.g., histone genes show a marked preference for codons enriched in A and T residues). If there is any evidence for such a bias in the gene of interest, alter your choice of codons accordingly.

4. Different mammalian tissues display different patterns of codon usage (Newgard et al. 1986). It would therefore be worthwhile to determine the codon usage in any genes that have already been cloned and are known to be expressed in the same tissue as your gene of interest.

5. At positions where the choice is between C and T and there is no strong codon preference, choose T. This rule is still somewhat controversial. There is good evidence indicating that rG:rU base pairs are more stable than rA:rC base pairs (Uhlenbeck et al. 1971). However, the extension to dG:dT base pairs (Wu 1972) has been both challenged (Smith 1983) and upheld (Martin et al. 1985).

6. Examine the sequence of the proposed nucleotide for regions of internal complementarity that might reduce hybridization efficiency. Although it is difficult to predict what level of internal complementarity might be significant, wherever possible avoid sequences that could form stable

duplexes under the conditions used for hybridization. The stability of duplexes can be predicted from the equation on page 11.52, assuming that there is a decrease of 1–1.5°C in the melting temperature of the duplex for each 1% of mismatch.

It is possible, and often desirable, to synthesize a small pool of 2–8 guessmers that contains all possible choices at certain positions. It is best to carry out this kind of limited substitution when an amino acid whose codon is highly degenerate separates two tracts containing only a few potential mismatches. In this way, it may be possible to generate a continuous sequence within the guessmer that is a perfect match for the target gene. Remember, however, that when using a mixture of oligonucleotides, the strength of the hybridization signal generated by the "correct" probe may be reduced. For guessmers labeled by phosphorylation or by filling of recessed ends (see below), an eightfold reduction in signal strength usually does not compromise the identification of clones of interest.

The length of guessmers that have been used successfully ranges from 32 to 86 nucleotides (see Table 11.1). Often the length of the oligonucleotide is dictated by the length of the available unambiguous amino acid sequence. However, even when a more extensive sequence of amino acids is available, there is little value in synthesizing an oligonucleotide longer than 70 nucleotides. Longer oligonucleotides are more expensive and difficult to synthesize and often must be constructed by ligating a set of overlapping shorter sequences and then propagating the product by cloning in a suitable vector (Itakura et al. 1977; Rossi et al. 1982; Jaye et al. 1983). It is therefore best to synthesize the longest probe possible, but not at the expense of including sequences that are unreliable or when it is necessary to construct the probe by ligation and/or cloning.

LABELING OF GUESSMERS

Oligonucleotides less than 70 nucleotides long are usually labeled by phosphorylation catalyzed by bacteriophage T4 polynucleotide kinase (see pages 11.31–11.32) or by primer extension using the Klenow fragment of *E. coli* DNA polymerase I and a specifically designed primer (see pages 11.40–11.44). However, longer oligonucleotides cloned into bacteriophage M13 vectors are almost always labeled in a primer-extension reaction using "universal" primer (see, e.g., Anderson and Kingston [1983] and Chapter 10, pages 10.19–10.24). The resulting labeled oligonucleotide is separated from the bacteriophage M13 template by chromatography through a column of Sepharose CL-4B equilibrated in 0.1 N NaOH or by electrophoresis through a denaturing polyacrylamide gel.

Alternatively, longer probes can be prepared from two separate oligonucleotides (corresponding to the coding and noncoding strands) that contain complementary sequences at their 3′ termini (see, e.g., Rossi et al. 1982; Derynck et al. 1984; Studencki and Wallace 1984; Ullrich et al. 1984b; Lauffer et al. 1985; Betsholtz et al. 1986) (Figure 11.2). These partially complementary oligonucleotides can be annealed and the protruding single-stranded regions filled in with ^{32}P-labeled nucleotides. If necessary, the 5′

termini can be labeled by phosphorylation before the filling reaction. Although simple to prepare, radiolabeled double-stranded guessmers have a significant disadvantage. Unless the sequence of the guessmer closely matches that of the gene, hybrids formed between the complementary strands of the probe may be significantly more stable than those formed between the probe and the gene. Over the course of the hybridization reaction, most of the label may become sequestered into hybrids formed between the complementary strands of the probe, and this can result in a dramatic reduction in the intensity of the signal.

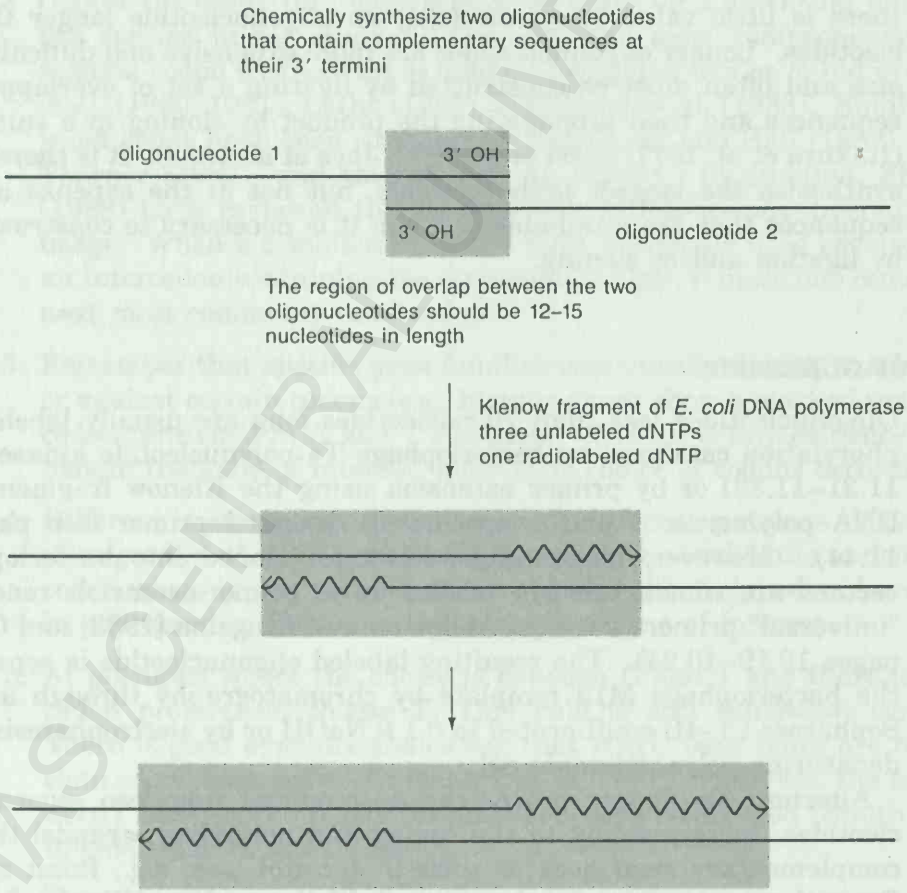
Chemically synthesize two oligonucleotides that contain complementary sequences at their 3′ termini
oligonucleotide 1
3′ OH
3′ OH
oligonucleotide 2
The region of overlap between the two oligonucleotides should be 12–15 nucleotides in length
Klenow fragment of E. coli DNA polymerase I
three unlabeled dNTPs
one radiolabeled dNTP

FIGURE 11.2
Generation of radiolabeled double-stranded oligonucleotide probes.

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Oligonucleotides That Contain a Neutral Base at Positions of Degeneracy

Probes of this type are used to reduce the degeneracy of pools of oligonucleotides. At positions where there is a choice of two, three, or four conventional bases, a "neutral" base can be used that pairs adequately with all four conventional bases. The deleterious effect of mismatching is greatly reduced because the base pairs formed between the neutral base and its conventional partners neither stabilize nor destabilize the remainder of the hybrid. The use of oligonucleotides that contain a neutral base at positions of ambiguity therefore has two advantages:

- *It removes much of the uncertainty in designing guessmers,* since it greatly reduces the possibility of creating mismatches that may compromise the hybridization of a single long oligonucleotide to its correct target sequences.
- *It allows the use of more stringent hybridization conditions.* Because the magnitude of the destabilizing influence of mismatched base pairs on hybrids formed between a conventional guessmer and its target sequence cannot be predicted, it is necessary to hybridize under conditions of low stringency. However, the incorporation of a neutral base whose base-pairing properties are known allows the melting temperature of the hybrid to be calculated more precisely. Hybridization can therefore be performed stringently.

The best neutral base currently available is inosine. It forms stable base pairs with all four conventional bases, and the strength of the pairing is approximately equal in each case (Martin et al. 1985; Ohtsuka et al. 1985; Takahashi et al. 1985). It is therefore better to use inosine rather than a conventional base at positions of three- or fourfold ambiguity (Martin et al. 1985). Furthermore, inosine can also be used in the third position of codons that code for the same amino acid and differ by A/G in the third position (e.g., Glu: GAA/GAG). However, the use of inosine in codons that differ by T/C in their third position is of less value. Mismatched T:G base pairs are somewhat more stable than I:G base pairs, and it would therefore seem to be better always to use T rather than I or C in the third position when faced with ambiguities of this type. However, under natural circumstances, C occurs much more frequently than T in the third position of such codons in mammalian nuclear genes (Lathe 1985). We therefore recommend that C be used in the third position unless the first nucleotide of the subsequent codon is G, in which case T should be used. However, the penalty for using inosine in place of T/C in the third position is quite small, and oligonucleotides containing inosine at all positions of ambiguity have in fact been used successfully to screen cDNA libraries (Ohtsuka et al. 1985; Takahashi et al. 1985; Jaye et al. 1986; Nagata et al. 1986) (Table 11.2).

A list of codons that are recommended when designing oligonucleotides that are to be used to screen human cDNA or genomic DNA libraries and that contain substitutions at positions of ambiguity in the genetic code is given below. The recommendations take into account the natural usage of codons

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in humans and the fact that the sequence CpG is underrepresented in human DNA.

1	2	3	4	6
Met ATG	Phe TTC[a]	Ile ATI	Val GTI	Leu CTI[c]
Trp TGG	Tyr TAC[a]		Pro CCI	Ser TCC[b,c]
	His CAC[a]		Thr ACI	Arg CGI[c]
	Gln CAI		Ala GCI	
	Asn AAC[a]		Gly GGI	
	Lys AAI			
	Asp GAT			
	Glu GAI			
	Cys TGC[a]			

[a]If the first nucleotide of the succeeding codon is G, use T in the third position.
[b]If the first nucleotide of the succeeding codon is G, use I in the third position.
[c]Try to avoid amino acids with six codon choices if at all possible.

Substituted probes are used in hybridization solvents containing sodium salts. There are no data yet available describing their behavior in solvents containing quaternary alkylammonium salts, which minimize the effects of base composition of nonsubstituted oligonucleotides on melting temperature (see pages 11.48–11.49).

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TABLE 11.2 Conditions Used to Screen Mammalian cDNA Libraries with Oligonucleotides Containing Inosine

Length	Number of inosine residues	Percent G + C	Hybridization conditions	Wash conditions	Gene	Reference
23	5	50	0.9 M Na^+ 45–50°C	0.94 M Na^+ 20°C	gastrin	Ohtsuka et al. (1985)
26	5	47	0.9 M Na^+ 45–50°C	0.94 M Na^+ 20°C	cholecystokinin	Ohtsuka et al. (1985)
30	3	44	0.9 M Na^+ 56°C	1.2 M Na^+ 56°C for 1.5 minutes	human granulocyte colony-stimulating factor	Nagata et al. (1986)
26	5	48	0.9 M Na^+ 55°C	1.2 M Na^+ 55°C for 1.0 minute	human cholecystokinin	Takahashi et al. (1985)
33	6	37	1.2 M Na^+ 42°C	0.4 M Na^+ 37°C	human endothelial cell growth factor	Jaye et al. (1986)
42	5	55	0.98 M Na^+ 20% formamide	0.04 M Na^+ 45°C	mouse macrophage-lymphocyte Fc receptor	Lewis et al. (1986)
43	6–8	48	1.6 M Na^+ 20% formamide	0.06 M Na^+ 42°C	human G_{α_s} signal transduction protein	Bray et al. (1986)

This table contains a representative sample of the conditions used to screen mammalian cDNA libraries with oligonucleotides containing inosine. It was not our intention to present a listing of all genes that have been isolated in this way.

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Purification and Radiolabeling of Synthetic Oligonucleotides

Almost all synthetic oligonucleotides are now prepared either individually or in pools by automated instruments sold by a variety of manufacturers. These instruments are capable of synthesizing nanomole amounts of oligonucleotides as long as 70–100 nucleotides. In addition, several companies will synthesize oligonucleotides of defined sequence to order. The minimum amount of an oligonucleotide synthesized by automatic machines (5–50 nmoles) is far in excess of the quantities usually required for hybridization, DNA sequencing, or site-directed mutagenesis.

Shorter oligonucleotides synthesized by modern instruments are often suitable for use without further purification; however, preparations of longer oligonucleotides may be contaminated by fragments that should be removed by polyacrylamide gel electrophoresis. This is essential when the oligonucleotide is to be used in primer-extension experiments to map the 5′ termini of mRNAs (see Chapter 7), is desirable when the oligonucleotide is to be used for site-directed mutagenesis (see Chapter 15), and is generally unnecessary when the oligonucleotide is to be used as a hybridization probe.

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PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES

The following methods are modified from procedures provided by T. Atkinson, M. Zoller, A. Cowie, G. Brown, and M. Smith (all pers. comm.).

Preparation of Synthetic Oligonucleotides

Oligonucleotides are usually supplied as a lyophilized powder.

1. In a sterile microfuge tube, dissolve the crude oligonucleotide (usually 1–10 nmoles) in 1 ml of sterile, filtered H_2O (Milli-Q or equivalent). Vortex the solution thoroughly. The solution is often slightly cloudy because of the presence of insoluble benzamides.

2. Centrifuge at 12,000*g* for 5 minutes at room temperature in a microfuge. Transfer the supernatant to a sterile microfuge tube.

3. Extract the solution three times in succession with 400 μl of 1-butanol (see Appendix E). Discard the upper (organic) phase after each extraction.

4. Evaporate the solution to dryness in a centrifugal evaporator (Savant SpeedVac or its equivalent). The tube should contain a yellowish pellet and a creamy-white powder.

5. Redissolve the pellet and powder in 200 μl of sterile, filtered H_2O.

6. Estimate the amount of oligonucleotide in the preparation as follows: Add 1 μl of the solution to 1 ml of H_2O. Mix well and read the OD_{260}. The absorbance should be 0.3–0.7.

   Recover the oligonucleotide by denaturing polyacrylamide gel electrophoresis as described on pages 11.23–11.28.

Amounts of oligonucleotides are often described in OD units. One OD corresponds to the amount of oligonucleotide in a 1-ml volume that results in an optical density of 1 in a 1-cm path-length cuvette. This corresponds to approximately 33 μg of oligonucleotide, although it varies for each particular oligonucleotide depending on its sequence. The concentration of an oligonucleotide of known sequence can be calculated since it is known that the extinction coefficient (in a 1-cm path-length cuvette) for each of the bases is

dGTP = 11.7 ml/μmole
dCTP = 7.3 ml/μmole
dATP = 15.4 ml/μmole
dTTP = 8.8 ml/μmole

For any given oligonucleotide, multiply the number of times each base appears by its extinction coefficient. Add the resulting four numbers to obtain the extinction coefficient (ϵ) for the entire oligonucleotide. The concentration (C) can then be calculated from the equation (for a 1-cm path-length cuvette):

$$OD = \epsilon \cdot C$$

More approximate estimates may be calculated as follows:

$$\frac{\text{total } OD_{260}}{10 \times \text{length of oligonucleotide}} = \mu\text{moles oligonucleotide present in the solution}$$

or

$$1\ OD_{260} \cong 33\ \mu\text{g/ml oligonucleotide}$$

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Note

If time is short, the extraction with 1-butanol can often be omitted without leading to problems. However, it is then not possible to use optical absorbance at 260 nm to estimate the amount of oligonucleotide in the crude preparation because of the presence of contaminants. If extraction with 1-butanol is to be omitted, dissolve the crude oligonucleotide in 200 μl of sterile, filtered H_2O. Centrifuge at 12,000*g* for 5 minutes at room temperature in a microfuge, and transfer the supernatant to a sterile microfuge tube. Load 10–30 μl of this solution onto each of six slots (1 cm) of a denaturing polyacrylamide gel as described on pages 11.23–11.28.

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1. Prepare the following stock solutions:

Acrylamide stock

acrylamide (DNA-sequencing grade)	190 g
N,N'-methylenebisacrylamide	10 g
H_2O to 250 ml	

Dissolve the chemicals by heating the solution to 37°C. Adjust the volume of the solution to 500 ml. Filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size), and store in dark bottles at room temperature.

Caution: Acrylamide is a potent neurotoxin and is absorbed through the skin. The effects of acrylamide are cumulative. Wear gloves and a mask when weighing powdered acrylamide and methylenebisacrylamide. Wear gloves when handling solutions containing these chemicals. Although polyacrylamide is considered to be nontoxic, it should be handled with care because of the possibility that it might contain small quantities of unpolymerized acrylamide.

Cheaper grades of acrylamide and bisacrylamide are often contaminated with metal ions. Stock solutions of acrylamide can easily be purified by stirring overnight with about 0.2 volume of monobed resin (MB-1, Mallinckrodt), followed by filtration through Whatman No. 1 paper.

During storage, acrylamide and bisacrylamide are slowly converted to acrylic acid and bisacrylic acid. This deamination reaction is catalyzed by light and alkali. Check that the pH of the acrylamide solution is 7.0 or less, and store the solution in dark bottles at room temperature. Prepare fresh solutions every few months.

5 × TBE

Tris base	54 g
boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
H_2O to 1 liter	

TBE is used at a working strength of 1 × (89 mM Tris-borate, 2 mM EDTA) for polyacrylamide gel electrophoresis. This is twice the strength usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for polyacrylamide gel electrophoresis are fairly small, and the amount of electric current passed through them is often considerable. 1 × TBE is required to provide adequate buffering power. The pH of the buffer should be ~8.3.

10% Ammonium persulfate

Ammonium persulfate	1 g
H_2O to 10 ml	

The solution may be stored at 4°C for several weeks.

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2. Prepare the glass plates and spacers for pouring the gel. If it is necessary to remove silicon residue from the plates, clean the plates with KOH/methanol, which is prepared by adding ~5 g of KOH pellets to 100 ml of methanol.

   Caution: Handle the KOH and the KOH/methanol solutions with great care. Use gloves and a face protector.

   Then wash the glass plates and spacers in warm detergent solution and rinse thoroughly in tap water, followed by deionized water. Hold the plates by the edges so that material from the gloves or oils from your hands do not become deposited on the working surfaces of the plates. Rinse the plates with ethanol and set them aside to dry. The plates must be free of grease spots to prevent air bubbles from forming in the gel.

   Treat one surface of each plate with a silicone solution. This prevents the gel from sticking tightly to both plates and reduces the possibility that the gel will tear when it is removed from the mold when electrophoresis is completed.

   To siliconize a plate, lay the plate on a pad of paper in a fume hood, and pour a small quantity of siliconizing fluid (e.g., Sigmacote) onto it. Wipe the fluid over the surface of the plate with a pad of Kimwipes, and then rinse the plate in deionized water. Dry the plate with a hairdryer. Wear gloves during siliconization.

3. There are many types of electrophoresis apparatus available commercially, and the arrangement of the glass plates and spacers differs slightly from manufacturer to manufacturer. In all cases, the aim is to form a watertight seal between the plates and the spacers so that the unpolymerized gel solution does not leak out. Typically, the two plates are of slightly different size and one of them is notched. Lay the larger (or unnotched) plate flat on the bench and arrange the spacers at each side parallel to the two edges (see Figure 11.3). A couple of *minute* dabs of petroleum jelly help to keep the spacer bars in position during the next steps. Lay the inner (notched) plate in position, resting on the spacer bars. Bind the entire length of the two sides and the bottom of the plates with gel-sealing tape (3M yellow electrical tape; BRL) to make a watertight seal. Take particular care with the bottom corners of the plates since these are the places where leaks often occur.

   Several other methods have been used to prevent leakage of acrylamide solution from assembled gel molds, including:
   - Sealing the edges with agarose
   - Inserting a plastic spacer into the open space at the bottom of the mold during polymerization
   - Sealing the bottom of the plate with a strip of filter paper impregnated with catalyzed acrylamide (Wahls and Kingzette 1988)

   We recommend that you use whichever of these methods you find to be most reliable with the particular type of gel mold that is available.

4. Prepare a polyacrylamide/urea gel (40 cm × 20 cm × 0.5 mm) (i.e., slightly thicker than the usual sequencing gel). Adjust the percentage of polyacrylamide in the gel according to the length of the oligonucleotide (see table below). Mix the components and warm the solution to 37°C to dissolve the urea. Filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size) and cool to room temperature.

Length of oligonucleotide	Percent polyacrylamide
25 nucleotides or less	19
25–40 nucleotides	15
40–100 nucleotides	12

	Amounts of reagents to cast gels of various concentrations (%)		
Reagents	**12%**	**15%**	**19%**
Urea (g)	31.5	31.5	31.5
Acrylamide stock (ml)	23.5	29.5	37.5
5× TBE (ml)	15	15	15
Water (ml)	14	8	0

5. Add 0.45 ml of a freshly prepared solution of 10% ammonium persulfate to the acrylamide solution. Mix well, being careful to avoid aeration of the solution.

6. Wearing gloves, perform the following manipulations over a tray so that any spilled acrylamide solution will not spread over the bench.

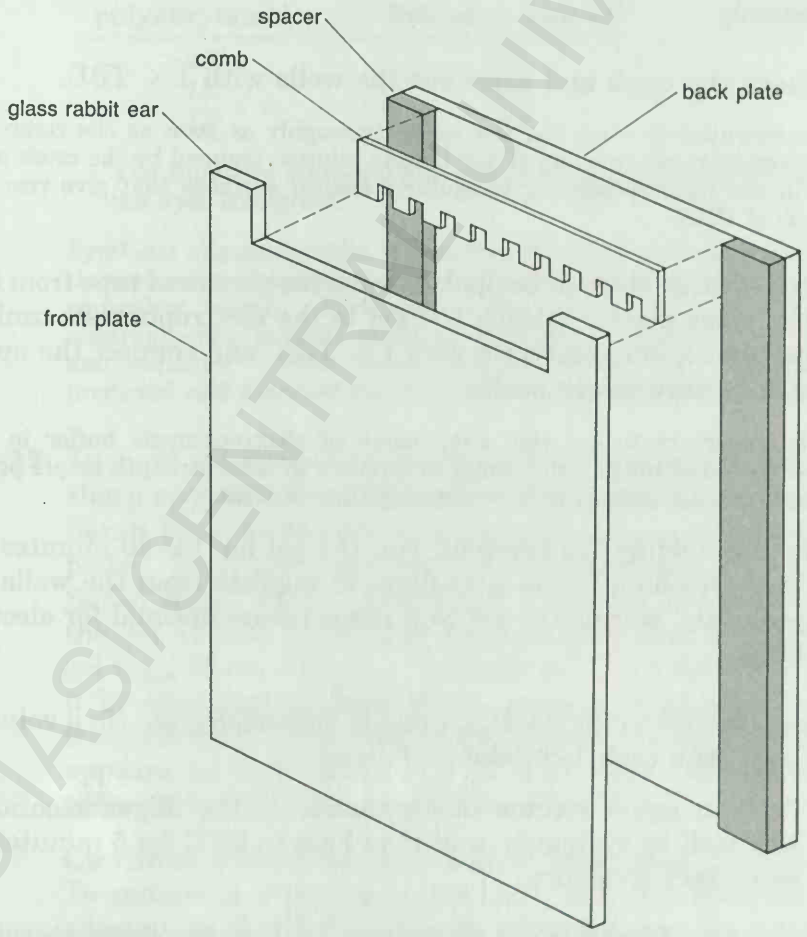

FIGURE 11.3

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a. Add 35 μl of TEMED (*N,N,N′,N′*-tetramethylethylenediamine). Mix the solution by swirling.

b. Draw the solution into the barrel of a 50-ml syringe. Invert the syringe and expel any air that has entered the barrel. Introduce the nozzle of the syringe into the space between the two glass plates. Expel the acrylamide solution from the syringe, filling the space almost to the top. If the plates were clean, there should be no trapped air bubbles, and if they were sealed well, no leaks. If air bubbles form, empty the gel mold and repour the gel after thoroughly recleaning the glass plates. Keep the remaining acrylamide solution at 4°C to reduce the rate of polymerization.

c. Lay the glass plates against a test-tube rack at an angle of ~10°. This decreases the chance of leakage and minimizes distortion of the gel.

7. Immediately insert the appropriate comb (1-cm slots are ideal), being careful not to allow air bubbles to become trapped under the teeth. Allow the acrylamide to polymerize for 30 minutes at room temperature, adding additional acrylamide solution if the gel retracts. When polymerization is complete, a schlieren pattern appears just beneath the teeth of the comb.

8. Remove the comb and rinse out the wells with 1 × TBE.

   It is essential to wash out the wells thoroughly as soon as the comb is removed. Otherwise, small amounts of acrylamide solution trapped by the comb will polymerize in the wells, producing irregularly shaped surfaces that give rise to distorted bands of DNA.

9. Using a razor blade or scalpel, remove the electrical tape from the bottom of the glass plates. Attach the gel to the electrophoresis tank. Fill the upper and lower reservoirs with 1 × TBE, and connect the apparatus to a high-voltage power pack.

   It is important to use the same batch of electrophoresis buffer in both of the reservoirs and in the gel. Small differences in ionic strength or pH produce buffer fronts that can greatly distort the migration of DNA.

   Before loading the samples, run the gel for 15–30 minutes at 1500 V. This causes ammonium persulfate to migrate from the wells and, more importantly, warms the gel to a temperature optimal for electrophoresis of DNA.

10. Load approximately 2 OD_{260} units of oligonucleotide (in a volume of 10 μl or less) onto each 1-cm slot as follows:

    a. Add an equal volume of formamide to the oligonucleotide solution. Mix well by vortexing, and then heat to 55°C for 5 minutes to disrupt secondary structure.

       It is not advisable to add conventional tracking dyes (bromophenol blue and/or xylene cyanol FF) to the loading buffer. These dyes (or contaminants in them)

may migrate at the same rate as the oligonucleotide and interfere with its detection by absorption of ultraviolet light (see step 12). If desired, 0.2% orange G can be included in the loading buffer. This dye migrates with the buffer front and does not interfere with detection of the oligonucleotide.

Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

b. Turn off the current and flush out the urea from the wells with 1 × TBE.

c. Load 5–6 slots with the heated oligomer. In an unused slot, load 5 μl of a 50:50 mixture of formamide and an aqueous solution of tracking dyes (0.05% xylene cyanol FF and 0.05% bromophenol blue).

11. Run the gel at 1500 V until the oligonucleotide has migrated approximately two thirds of the length of the gel. If necessary, a metal plate can be clamped to the gel to help eliminate curvature of the bands caused by thermal gradients. The position of the oligonucleotide may be estimated from the positions of the tracking dyes:

Percent polyacrylamide	Xylene cyanol FF[a]	Bromophenol blue[a]
19	22	6
15	30	9–10
12	40	~15

[a]The numbers represent the approximate length of oligonucleotides with which the dyes comigrate.

Synthetic oligonucleotides that carry a hydroxyl residue at their 5′ terminus migrate through polyacrylamide gels slightly slower than phosphorylated oligonucleotides of equivalent length. Furthermore, for reasons that are not fully understood, the electrophoretic mobility of oligonucleotides is dependent on their base composition and sequence. Thus, there may not be an exact correspondence between the predicted and observed positions of the oligonucleotide in the polyacrylamide gel.

12. Turn off the current and dismantle the gel apparatus. Use the edge of a sharp spatula to lever the upper glass plate from the gel. Place a piece of Saran Wrap on the gel, which should remain attached to the lower glass plate. Turn the glass plate over so that the gel is transferred to the Saran Wrap. Place a fluorescent thin-layer chromatographic plate under the gel. (These plates may be purchased from Brinkmann: Merck Silica gel F_{254}, 20 cm × 20 cm.) Take the gel into a darkened room and examine it by illumination from above, using a hand-held, long-wavelength ultraviolet lamp. The DNA in the gel absorbs the ultraviolet radiation and appears as dark bands against a uniform fluorescent background contributed by the chromatographic plate.

Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

13. Recover the desired oligonucleotide, which should be the slowest-migrating band, by chopping out each track of the gel with a sharp, clean scalpel. Avoid taking material smaller than the desired oligonucleotide.

14. Transfer the gel slices to three or four microfuge tubes. Add 1 ml of elution buffer to each tube. Crush the slices with a disposable pipette tip, using a circular motion and pressing against the sides of the tubes. Seal the tubes well. Incubate for 12 hours at 37°C in a shaker incubator.

Oligonucleotide elution buffer

0.1% SDS
0.5 M ammonium acetate
10 mM magnesium acetate

15. Centrifuge the tubes at 12,000*g* for 5 minutes at room temperature in a microfuge. Transfer the pooled supernatants to a 5-ml disposable syringe and pass them through a Millex HV filter (Millipore, 0.45-micron pore size). Collect the effluent in a 15-ml polypropylene tube.

Oligonucleotides can also be recovered from polyacrylamide gels by the electroelution procedure described by Zassenhaus et al. (1982). A device for this purpose is commercially available (ISCO Electrophoretic Concentrator).

16. Isolate oligonucleotides from the filtered solution as described on pages 11.29–11.30.

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Isolation of Oligonucleotides by Reversed-phase Chromatography on a Silica Gel

Isolate oligonucleotides from the filtered solution (step 15, page 11.28) either by reversed-phase chromatography on a silica gel as described below or by precipitation with ethanol. Precipitation with ethanol works efficiently if the volume of the filtered solution is reduced by repeated extractions with 1-butanol (see Appendix E) and if the oligonucleotide is longer than 18 nucleotides. Reversed-phase chromatography is more rapid, works efficiently with oligonucleotides of all lengths, and yields preparations of higher purity. The oligonucleotide adsorbs to the column when the polarity of the solvent is high (e.g., aqueous buffers) and elutes from it when the polarity of the solvent is reduced (e.g., a mixture of methanol and H_2O).

Note: The following procedure is simple and works well for purifying and concentrating newly synthesized oligonucleotides. It cannot be used to separate [γ-^{32}P]ATP from an oligonucleotide following a phosphorylation reaction. A slightly more complicated procedure that can be used to purify and concentrate synthetic oligonucleotides as well as remove [γ-^{32}P]ATP following a phosphorylation reaction is given on page 11.39.

1. Prepare a Sep-Pak C_{18} reversed-phase column as follows:

   a. Attach the barrel of a disposable 10-ml polypropylene syringe to the longer end of a Sep-Pak C_{18} column (Waters).

   b. Add 10 ml of high-performance liquid chromatography (HPLC)-grade acetonitrile to the barrel and slowly push it through the column with the plunger of the syringe.

      Caution: Acetonitrile should be handled in a chemical hood, wearing gloves and goggles. Inhalation of vapors may be harmful and contact with skin or eyes may cause irritation. Acetonitrile is also flammable. Keep away from heat and flames.

   c. *Remove the syringe from the Sep-Pak column.* Take the plunger out of the barrel. Reattach the barrel to the column. (This is done to prevent air being sucked back into the column.)

   d. Add 10 ml of sterile, filtered H_2O (Milli-Q or equivalent) to the barrel and slowly push it through the column with the plunger. Repeat step c.

   e. Add 2 ml of 10 mM ammonium acetate to the barrel and push it slowly through the column. Again remove the syringe, remove the barrel, and reattach the barrel to the column. The column is now ready for use.

2. Add the filtered solution to the barrel and slowly push it through the column with the plunger. Collect the effluent in a sterile 50-ml polypropylene tube. Repeat step 1c.

3. Add 10 ml of sterile, filtered H_2O (Milli-Q or equivalent) to the barrel and push it slowly through the column with the plunger. Repeat step 1c. Repeat the water wash twice.

4. Elute the oligonucleotide three times with 1 ml of a 60:40 mixture of methanol:H_2O. Repeat step 1c after each elution. Collect each effluent in a separate microfuge tube. Read the OD_{260} of the solution in each of the three microfuge tubes, using the methanol:H_2O mixture as a blank. More than 90% of the oligonucleotide applied to the column should elute in the first fraction.

5. Evaporate the solution containing the oligonucleotide to dryness in a centrifugal evaporator (Savant SpeedVac or its equivalent).

6. Redissolve the oligonucleotide in a total volume of 200 μl of sterile, filtered H_2O (Milli-Q or equivalent).

7. Dilute 5 μl of the solution into 1 ml of H_2O. Read the OD_{260} of the diluted sample, and calculate the amount of oligonucleotide present in the original solution.

   Amounts of oligonucleotides are often described in OD units. One OD corresponds to the amount of oligonucleotide in a 1-ml volume that results in an optical density of 1 in a 1-cm path-length cuvette. This corresponds to approximately 33 μg of oligonucleotide, although it varies for each particular oligonucleotide depending on its sequence. The concentration of an oligonucleotide of known sequence can be calculated since it is known that the extinction coefficient (in a 1-cm path-length cuvette) for each of the bases is

   dGTP = 11.7 ml/μmole
   dCTP = 7.3 ml/μmole
   dATP = 15.4 ml/μmole
   dTTP = 8.8 ml/μmole

   For any given oligonucleotide, multiply the number of times each base appears by its extinction coefficient. Add the resulting four numbers to obtain the extinction coefficient (ϵ) for the entire oligonucleotide. The concentration (C) can then be calculated from the equation (for a 1-cm path-length cuvette):

   $$OD = \epsilon \cdot C$$

   More approximate estimates may be calculated as follows:

   $$\frac{\text{total } OD_{260}}{10 \times \text{length of oligonucleotide}} = \mu\text{moles oligonucleotide present in the solution}$$

   or

   $$1\ OD_{260} \cong 33\ \mu\text{g/ml oligonucleotide}$$

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LABELING OF SYNTHETIC OLIGONUCLEOTIDES BY PHOSPHORYLATION WITH BACTERIOPHAGE T4 POLYNUCLEOTIDE KINASE

Synthetic oligonucleotides are synthesized without a phosphate group at their 5′ termini and are therefore easily labeled by transfer of the γ-^{32}P from [γ-^{32}P]ATP using the enzyme bacteriophage T4 polynucleotide kinase. If the reaction is carried out efficiently, the specific activity of such probes can be as high as the specific activity of the [γ-^{32}P]ATP itself. The reaction described below is designed to label 10 pmoles of an oligonucleotide to high specific activity. Labeling of different amounts of oligonucleotide can easily be achieved by increasing or decreasing the size of the reaction, keeping the concentrations of all components constant.

1. Set up a reaction mixture as follows:

oligonucleotide (10 pmoles/μl)	1.0 μl
10 × bacteriophage T4 polynucleotide kinase buffer	2.0 μl
[γ-^{32}P]ATP (sp. act. 5000 Ci/mmole; 10 mCi/ml in aqueous solution) (10 pmoles)	5.0 μl
H_2O	11.4 μl

10 × Bacteriophage T4 polynucleotide kinase buffer

0.5 M Tris · Cl (pH 7.6)
0.1 M $MgCl_2$
50 mM dithiothreitol
1 mM spermidine HCl
1 mM EDTA (pH 8.0)

Mix well, and place 0.5 μl of the reaction mixture in a tube containing 10 μl of 10 mM Tris · Cl (pH 8.0) and set aside to use in step 3.

The reaction contains equal concentrations of [γ-^{32}P]ATP and oligonucleotide, and only 50% of the radiolabel is transferred to the oligonucleotide. The *efficiency of transfer* can be improved by increasing the concentration of oligonucleotide in the reaction by a factor of 10. This results in transfer of approximately 90% of the radiolabel to the oligonucleotide. However, the *specific activity* of the oligonucleotide is reduced by a factor of approximately 5. To label an oligonucleotide to the highest specific activity:

a. Increase the concentration of [γ-^{32}P]ATP in the reaction by a factor of three (i.e., use 15 μl of radiolabel and decrease the volume of H_2O to 1.4 μl)
b. Decrease the concentration of oligonucleotide to 3 pmoles.

Under these circumstances, only about 10% of the radiolabel is transferred, but virtually every molecule of oligonucleotide becomes radiolabeled.

2. Add 8 units (~1 μl) of bacteriophage T4 polynucleotide kinase to the reaction mixture. Mix well, and incubate for 45 minutes at 37°C. Place another 0.5-μl aliquot of the reaction in a second tube containing 10 μl of 10 mM Tris · Cl (pH 8.0). Heat the remainder of the reaction for 10 minutes at 68°C to inactivate the bacteriophage T4 polynucleotide kinase.

3. Determine the efficiency of transfer of ^{32}P to the oligonucleotide and estimate its specific activity as follows (Stawinski et al. 1977; Narang et al. 1980):

   a. Cut a strip of cellulose impregnated with polyethyleneimine (Polygram CEL 300PEI; Brinkmann) about 5 cm wide and 15 cm long. With a soft-lead pencil, draw a fine line across the strip about 2.5 cm from one end, and make two small marks about one inch apart on the line.

   b. Spot 0.5 μl of each of the aliquots set aside in steps 1 and 2 at the marks, and place the strip vertically in a chromatography jar so that the radioactive samples are at the lower end of the strip. The bottom of the strip should dip into a petri dish filled to a level of approximately 0.5 cm with developing buffer (0.5 M ammonium bicarbonate). Cover the jar and allow the chromatograph to develop until the solvent front has migrated approximately 4–5 inches.

   c. Wrap the strip of polyethyleneimine-cellulose in Saran Wrap and autoradiograph, or cut the strip horizontally into thin (0.25-cm) sections and measure the amount of radioactivity in each section in a scintillation counter.

      Oligonucleotides will remain at the origin, whereas ATP and inorganic phosphate will migrate in the same direction as the solvent (inorganic phosphate migrates slightly slower than the solvent front and ATP is approximately equidistant between the origin and the inorganic phosphate). Thus, the transfer of phosphate from [γ-^{32}P]ATP to the oligonucleotide will result in the appearance of radioactivity at the origin. By measuring the amount of radioactivity at the origin and on the total strip, the percentage of radiolabel transferred from [γ-^{32}P]ATP to the oligonucleotide can be calculated. The specific activity of the probe can then be determined on the basis of the molar quantities of oligonucleotide and [γ-^{32}P]ATP in the reaction. Under the conditions described above, approximately 50% of the radioactivity should be transferred to the oligonucleotide, resulting in a specific activity of approximately 2500 Ci/mmole.

   The transfer of radiolabel to the oligonucleotide can also be monitored by adsorption to DE-81 filters. The oligonucleotide binds tightly to the positively charged filters, whereas unincorporated radiolabel is removed by repeated washing with a solution of sodium phosphate. See Appendix E for details.

4. If the specific activity of the probe is acceptable, proceed to "Purification of Radiolabeled Synthetic Oligonucleotides" on page 11.33. If the specific activity is too low, add an additional 8 units of enzyme, continue incubation for a further 30 minutes at 37°C (i.e., a total of 75 minutes), heat the reaction for 10 minutes at 68°C to inactivate the enzyme, and analyze the products of the reaction again.

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PURIFICATION OF RADIOLABELED SYNTHETIC OLIGONUCLEOTIDES

The extent to which radiolabeled oligonucleotides need to be purified varies according to their eventual use. If they are to be used only as probes in hybridization experiments, complete removal of unincorporated radiolabel is generally unnecessary. However, to reduce background, it is advisable to separate the bulk of the unincorporated radioactivity from the oligonucleotide. This can be achieved rapidly by precipitation with ethanol if the oligonucleotide is more than 18 nucleotides in length or by precipitation with cetylpyridinium bromide if the oligonucleotide is shorter. If the oligonucleotide is to be used in an enzymatic reaction (e.g., primer extension), it may be necessary to use more stringent methods to remove the last traces of unincorporated radioactivity (e.g., chromatography through Bio-Gel P-60, chromatography on a Sep-Pak C_{18} column, or electrophoresis through a 19% polyacrylamide gel). Each of these procedures is described in detail in this section. Purification by electrophoresis through polyacrylamide gels is essentially as described beginning on page 11.43, step 5.

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Purification of Radiolabeled Oligonucleotides by Precipitation with Ethanol

1. After inactivation of the bacteriophage T4 polynucleotide kinase by heat (step 2, page 11.31, or step 4, page 11.32), add 40 μl of H_2O to the tube. Mix well, and then add 240 μl of a 5 M solution of ammonium acetate. Mix well again, and then add 750 μl of ice-cold ethanol. Mix well, and store for 30 minutes at 0°C.

2. Recover the radiolabeled oligonucleotide by centrifugation at 12,000*g* for 20 minutes at 0°C in a microfuge. Using an automatic pipetting device equipped with a disposable tip, carefully remove all of the supernatant fluid (which contains most of the unincorporated [γ-^{32}P]ATP and any free ^{32}P generated during the phosphorylation reaction).

3. Add 500 μl of 80% ethanol, vortex briefly, and recentrifuge. Using an automatic pipetting device equipped with a disposable tip, carefully remove the supernatant (which should contain appreciable amounts of radioactivity). Stand the open tube behind a Plexiglas screen until all of the residual ethanol has evaporated.

4. Redissolve the radiolabeled oligonucleotide in 100 μl of TE (pH 7.6). The radiolabeled oligonucleotide may be stored for a few days at −20°C. However, during prolonged storage, decay of ^{32}P causes radiochemical damage that can impair the ability of the oligonucleotide to hybridize to its target sequence.

Note

This method of purification works best for oligonucleotides that are more than 18 nucleotides long. Shorter oligonucleotides, which are not always quantitatively precipitated by this procedure, can be purified by one of the methods given on the following pages.

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Purification of Radiolabeled Oligonucleotides by Precipitation with Cetylpyridinium Bromide

This method, which was originally devised to recover DNA eluted from hydroxyapatite columns in phosphate buffers (Geck and Nász 1983), can also be used to precipitate radiolabeled nucleic acids, including oligonucleotides, from labeling reactions. The nucleic acids are precipitated from aqueous solution with the cationic detergent cetylpyridinium bromide (CPB). The detergent is then removed from the precipitate with ethanol (in which the nucleic acids are insoluble), and the nucleic acids are finally redissolved in the buffer of choice. The method is extremely rapid and efficient and works well with DNA (e.g., from nick-translation reactions), RNA (e.g., from in vitro transcription reactions with bacteriophage SP6, T3, or T7 polymerase), and oligonucleotides (e.g., from phosphorylation reactions). However, it suffers from one possible disadvantage—the necessity to use carrier DNA. For most purposes, the presence of the unlabeled carrier DNA in the final preparation of radiolabeled oligonucleotide is either irrelevant or an advantage (e.g., if the radiolabeled oligonucleotide is to be used as a probe). However, in certain specialized cases (e.g., when a radiolabeled oligonucleotide is to be used in primer-extension reactions), the presence of carrier DNA may cause complications. In these cases, we recommend that the oligonucleotide be purified by electrophoresis through a 19% polyacrylamide gel (see page 11.43), by chromatography through Bio-Gel P-60 (see page 11.37), or by Sep-Pak column chromatography (see page 11.39).

The following stock solutions are required:

1% CPB

Dissolve 1 g of CPB (Sigma) in 100 ml of H_2O. To speed dissolution, heat the solution on a hot plate and agitate with a magnetic stirrer. After all of the detergent has dissolved, cool the solution to room temperature and filter it through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size).

0.5 M EDTA-Tris (pH 6.0)

Add 0.1 mole of EDTA (free acid m.w. = 292.2) to 60 ml of H_2O. While stirring the solution, slowly add Tris base (powder) until the pH of the solution reaches 6.0. By this stage, the concentration of Tris will be approximately 1.2 M. Adjust the volume of the solution to 200 ml with H_2O. This unconventional method of preparing this solution may be essential for efficient precipitation of nucleic acids with cationic detergents (Sibatani 1970).

EDTA-Tris-DNA

Dissolve carrier DNA (e.g., salmon sperm DNA) at a concentration of 50 μg/ml in 50 mM EDTA-Tris (pH 6.0) (i.e., a 1:10 dilution of the 0.5 M stock solution). The DNA that is used as carrier should be of fairly uniform size and should not interfere with the hybridization of the radiolabeled nucleic acid to its target DNA.

For most purposes, sonicated or fractionated salmon sperm DNA works well (sonicated salmon sperm DNA 600–5000 bp in length is available from Pharmacia).

The following protocol, which was provided by R. MacDonald and G. Swift (pers. comm.), is C. Szent-Gyorgi's modification of the original method of Geck and Nász (1983).

1. Add 5–10 volumes of the EDTA-Tris-DNA solution to a microfuge tube containing the solution of radiolabeled oligonucleotide that is to be precipitated. Mix well. For precipitation to be efficient, it is important that the ionic composition of the final mixture be predominantly that of the EDTA-Tris-DNA solution. The volume of the mixture and the concentration of the radiolabeled oligonucleotide do not affect the efficiency of the procedure.

2. Add sufficient 1% CPB to bring the concentration of the detergent in the mixture to 0.1%. Mix well.

3. Place the tube in a dry-ice/methanol bath until the mixture is frozen. Remove the tube from the bath, and allow the mixture to thaw at room temperature.

4. Centrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge. Using a pasteur pipette, carefully remove all of the fluid from the tube. Add 500 μl of distilled H_2O, vortex for 20 seconds, and recentrifuge.

5. Carefully remove all of the fluid from the tube, and repeat the washing procedure.

6. Carefully remove all of the fluid from the tube, and add 500 μl of a solution consisting of 80% ethanol and 20% 0.1 M sodium acetate (pH 5.2). Vortex for 15 minutes, and centrifuge at 12,000*g* for 2 minutes at room temperature in a microfuge.

7. Repeat step 6.

8. Carefully remove all of the fluid from the tube, and stand the open tube on the bench until the last visible traces of ethanol have evaporated. Dissolve the precipitated oligonucleotide in 20 μl of TE (pH 7.6).

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1. After inactivation of the bacteriophage T4 polynucleotide kinase by heat (step 2, page 11.31, or step 4, page 11.32), add 30 μl of 20 mM EDTA (pH 8.0) to the reaction. Store at 0°C while preparing a column of Bio-Gel P-60.

2. Prepare a column of Bio-Gel P-60 (Bio-Rad, fine grade) in a sterile pasteur pipette. Equilibrate the slurry of Bio-Gel P-60 supplied by the manufacturer in 10 volumes of 10 mM Tris · Cl (pH 8.0), 0.1% SDS. Place a sterile glass-wool plug in a sterile pasteur pipette as shown in Figure 11.4. Fill the pipette with the slurry. The column will form rapidly as the gel matrix settles under gravity and the buffer drips from the pipette. Add additional slurry until the packed column fills the pipette from the plug of glass wool to the constriction near the top of the pipette. Wash the column with 3 ml of buffer containing 10 mM Tris · Cl (pH 8.0), 0.1% SDS. Do not

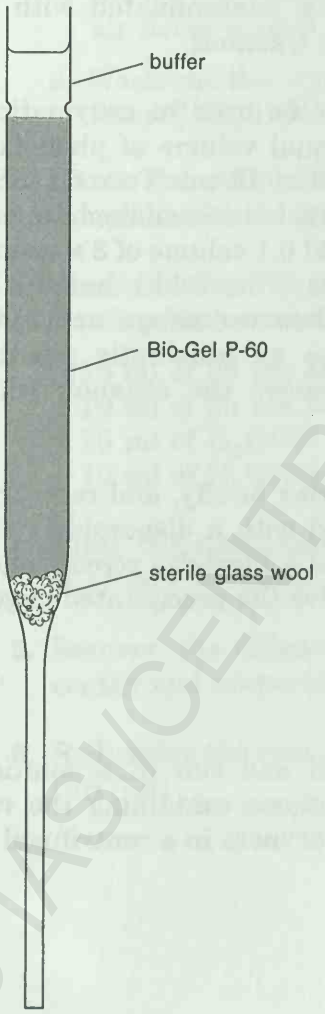

FIGURE 11.4
Pasteur pipettes can be used for column chromatography as described in step 2 above.

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allow the column to run dry. If necessary, the bottom of the column can be sealed by wrapping a piece of Parafilm around the bottom of the pipette.

3. Using a pipette, remove excess buffer from the top of the column, and then rapidly load the radiolabeled oligonucleotide (in a volume of 100 μl or less) on the column. Immediately after the sample has entered the column, add 100 μl of buffer to the top of the column. As soon as this has entered the column, fill the pipette with buffer. Replenish the buffer as necessary so that the column never runs dry.

4. Use a hand-held minimonitor to follow the progress of the radiolabeled oligonucleotide. When the radioactivity first starts to elute from the column, begin collecting two-drop fractions into microfuge tubes.

5. When nearly all of the radioactivity has eluted from the column, analyze approximately 0.5 μl of every other fraction either by chromatography on a strip of polyethyleneimine-cellulose (see page 11.32) or by adsorption to DE-81 filters (see Appendix E). Identify those fractions containing radiolabeled oligonucleotide that are not contaminated with unincorporated [γ-^{32}P]ATP. Pool the appropriate fractions.

6. If the radiolabeled oligonucleotide is to be used in enzymatic reactions, extract the pooled fractions with an equal volume of phenol:chloroform. Reextract the organic phase with 50 μl of 10 mM Tris·Cl (pH 8.0), and combine the two aqueous phases. Extract the combined aqueous phases with an equal volume of chloroform. Add 0.1 volume of 3 M sodium acetate (pH 5.2), mix well, and add 3 volumes of ice-cold ethanol. Freeze the sample for 30 minutes at 0°C, and then centrifuge at 12,000*g* for 20 minutes at 4°C in a microfuge. Use an automatic pipetting device equipped with a disposable tip to remove the ethanol (which should contain very little radioactivity).

7. Add 500 μl of ice-cold 80% ethanol, vortex briefly, and recentrifuge. Use an automatic pipetting device equipped with a disposable tip to remove the ethanol. Store the open tube behind a Plexiglas screen, and allow the residual ethanol to evaporate. Redissolve the precipitated oligonucleotide in 20 μl of H_2O and store at −20°C.

Note

The Bio-Gel P-60 column can be poured and run in a solution of 0.1% ammonium bicarbonate. The pooled fractions containing the radiolabeled oligonucleotide can then be evaporated to dryness in a centrifugal evaporator (Savant SpeedVac or its equivalent).

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Purification of Radiolabeled Oligonucleotides by Chromatography on a Sep-Pak C_{18} Column

The following is a modification of procedures described by Lo et al. (1984), Sanchez-Pescador and Urdea (1984), and Zoller and Smith (1984).

1. Prepare a Sep-Pak C_{18} reversed-phase column as follows:

   a. Attach a polypropylene syringe containing 10 ml of high-performance liquid chromatography (HPLC)-grade acetonitrile to a Sep-Pak C_{18} column (Waters).

      Caution: Acetonitrile should be handled in a chemical hood, wearing gloves and goggles. Inhalation of vapors may be harmful and contact with skin or eyes may cause irritation. Acetonitrile is also flammable. Keep away from heat and flames.

   b. Apply the acetonitrile to the matrix.

   c. *Remove the syringe from the Sep-Pak column.* Take the plunger out of the barrel. Reattach the barrel to the column. (This is done to prevent air being sucked back into the column.)

   d. Wash out the organic solvent two times with a 10-ml aliquot of sterile, filtered H_2O (Milli-Q or equivalent). Repeat step c after each wash.

2. Dilute the radiolabeled sample to 1.5 ml with H_2O, and apply the whole sample to the column through the syringe.

3. Wash the Sep-Pak column with the following four solutions. Repeat step 1c after each wash.

   10 ml of 25 mM ammonium bicarbonate (pH 8.0)
   10 ml of 25 mM ammonium bicarbonate/5% acetonitrile
   10 ml of H_2O/5% acetonitrile
   10 ml of H_2O/5% acetonitrile

4. Elute the radiolabeled oligonucleotide three times with a 1-ml aliquot of H_2O/30% acetonitrile. Repeat step 1c after each elution.

5. Recover the oligonucleotide by evaporating the eluate to dryness in a centrifugal evaporator (Savant SpeedVac or its equivalent).

6. Redissolve the radiolabeled oligonucleotide in a small volume (10 μl) of TE (pH 7.6).

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LABELING OF SYNTHETIC OLIGONUCLEOTIDES USING THE KLENOW FRAGMENT OF *E. coli* DNA POLYMERASE I

In some cases (e.g., when using oligonucleotides as probes in Southern hybridizations), it is important to radiolabel to the highest possible specific activity. At best, phosphorylation results in the incorporation of one atom of ^{32}P per molecule of oligonucleotide. However, probes of higher specific activities can be obtained using the Klenow fragment of *E. coli* DNA polymerase I to synthesize a strand of DNA complementary to the synthetic oligonucleotide (Studencki and Wallace 1984; Ullrich et al. 1984b) (Figure 11.5). A short primer is hybridized to an oligonucleotide template whose sequence is the complement of the desired radiolabeled probe. The primer is then extended using the Klenow fragment of *E. coli* DNA polymerase I to incorporate [α-^{32}P]dNTPs in a template-directed manner. After the reaction, the template and product are separated by denaturation followed by electrophoresis through a polyacrylamide gel under denaturing conditions. With this method, it is possible to generate oligonucleotide probes that contain several radioactive atoms per molecule of oligonucleotide and to achieve

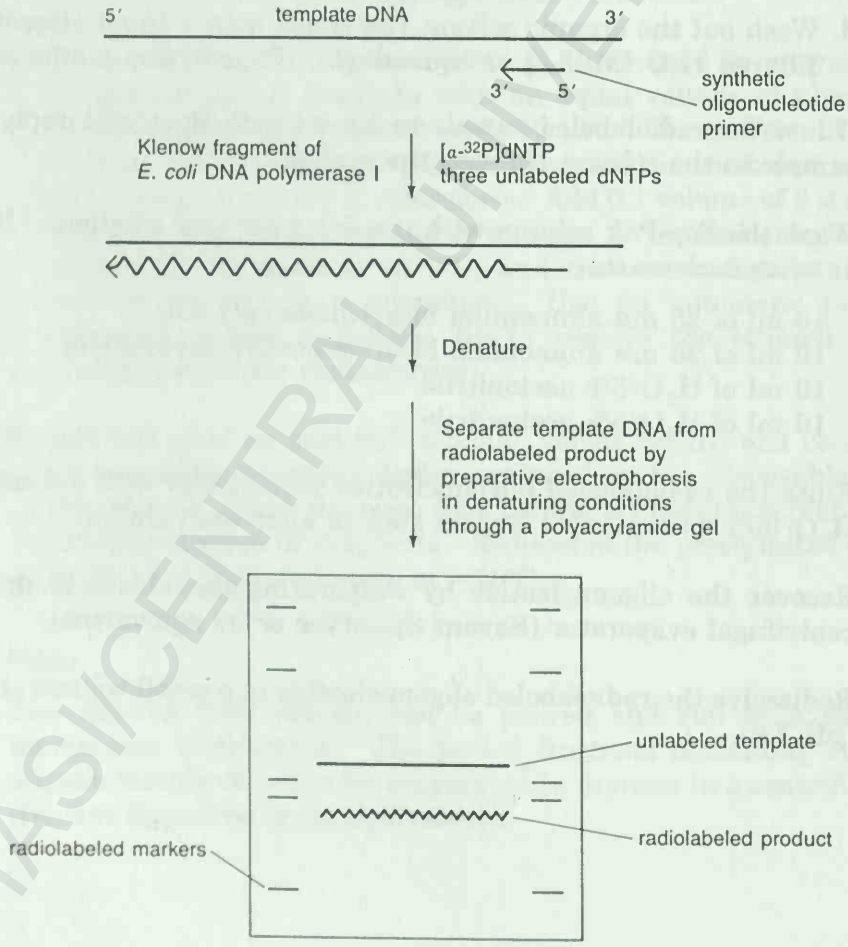

FIGURE 11.5
Labeling of synthetic oligonucleotides using the Klenow fragment of *E. coli* DNA polymerase I (see text for details).

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specific activities as high as 2×10^{10} cpm per microgram of probe. In a modification of the procedure, longer probes can be synthesized from two oligonucleotides that contain complementary sequences at their 3′ termini (Ullrich et al. 1984a) (see Figure 11.2). Both strands are extended, resulting in a duplex that may be considerably longer than either of the two original oligonucleotides (see pages 11.15–11.16).

When planning these sorts of reactions, it is important to consider the following points:

- *The specific activity of the [α-^{32}P]dNTPs in the reaction.* The specific activity of a dNTP whose α-phosphate has been completely substituted by ^{32}P is approximately 9000 Ci/mmole. Preparations with lower specific activities contain a mixture of both ^{32}P-labeled and unlabeled molecules. Thus, if a single radiolabeled dNTP with a specific activity of 3000 Ci/mmole is included in the synthetic reaction and there are only three positions at which that nucleotide can be incorporated into the final product, an average of only one atom of ^{32}P will be present in each molecule of probe (i.e., its specific activity will be approximately equal to that of a probe labeled by bacteriophage T4 polynucleotide kinase). Knowing the sequence of the desired probe and the specific activity of the available precursors, it is possible to predict its specific activity if one, two, three, or all four [α-^{32}P]dNTPs are included in the reaction.

- *The concentration of dNTPs in the reaction.* Polymerization of nucleotides will not proceed efficiently unless the concentration of each dNTP in the reaction is 1 μM or greater. It is therefore important to ensure that the concentration of substrates never drops below 1 μM (~0.66 ng/μl) as the reaction proceeds. Calculate the total quantity of each dNTP that would be incorporated into the probe, assuming that all of the single-stranded sequences in the reaction are used as templates. The total amount of each dNTP in the reaction should be the sum of the amount that could be incorporated into the probe plus 0.66 ng/μl.

- *The primer must be of sufficient length and specificity to bind to the template and promote synthesis at the appropriate position.* Primers used for this purpose are usually 7–9 nucleotides long and are complementary to sequences at or close to the 3′ terminus of the template oligonucleotide. Because the stability of such short hybrids is difficult to predict, the ratio of primer to template that gives the maximum yield of full-length probe should be determined empirically before embarking on large-scale reactions.

- *The final product is a double-stranded fragment of DNA whose length is equal to or slightly less than that of the template (depending on the location of the sequences complementary to the primer).* To be maximally effective as a probe, the unlabeled template strand must be efficiently separated from the complementary radiolabeled product; otherwise, the two complementary strands will anneal to each other and hybridization to the desired target sequence will be suppressed. For oligonucleotides less than 30 nucleotides in length, electrophoresis through a 19% polyacrylamide gel is the most effective method to separate the complementary strands. The

efficiency of the separation is improved if the lengths of the two strands are not exactly the same. Whenever possible, therefore, the primer should be designed so that it is complementary to the *subterminal* nucleotides of the template. If the primer cannot hybridize to the 2–3 nucleotides at the 3′ terminus of the template, the radiolabeled product will be shorter than the unlabeled template and will separate from it more effectively during electrophoresis. However, even when their lengths are identical, there is a good chance that the template and product strands will separate to some extent during electrophoresis since the rate of migration of single-stranded nucleic acids through gels is dependent not only on their length, but also on their base composition and sequence. The extent of separation can often be increased either by phosphorylating one of the two strands (template or primer) with nonradioactive ATP before synthesis of the probe (see page 11.31) or by leaving the dimethoxytrityl group attached to the 5′ terminus of the primer (Studencki and Wallace 1984). Because it is impossible to predict which method will be most effective for a given oligonucleotide, it is usually necessary to carry out a series of trial experiments to determine in each case the efficiency with which the product can be separated from the template.

1. Mix in a microfuge tube the calculated amounts of [α-^{32}P]dNTPs necessary to achieve the desired specific activity and sufficient to allow complete synthesis of all template strands. Remember that the concentration of dNTPs should not be less than 1 μM at any stage during the reaction (see above).

   To keep the concentration of reagents high, the reaction should be carried out in as small a volume as possible. It is therefore better to use as substrates radiolabeled dNTPs supplied in ethanol/water rather than those supplied in buffered aqueous solvents. Appropriate volumes of the ethanolic [α-^{32}P]dNTPs can be mixed and evaporated to dryness in the microfuge tube that will be used to carry out the reaction.

2. Add to the tube the appropriate amounts of primer and template DNAs. To guarantee efficient priming, the primer should be in three- to tenfold molar excess over the template.

3. Add 0.1 volume of 10 × Klenow buffer. Mix well.

   10 × Klenow buffer

   0.4 M potassium phosphate (pH 7.5)
   66 mM $MgCl_2$
   10 mM β-mercaptoethanol

4. Add 2–4 units of the Klenow fragment of *E. coli* DNA polymerase I per 5 μl of reaction volume. Mix well. Incubate for 2–3 hours at 4°C. If desired, the progress of the reaction may be monitored by removing small (0.1-μl) aliquots and measuring the proportion of radioactivity that has become precipitable with 10% trichloroacetic acid (TCA) (see Appendix E).

5. Dilute the reaction with an equal volume of gel-loading buffer, heat to 80°C for 3 minutes, and load the entire sample on a denaturing polyacrylamide gel (i.e., cast with 7 M urea).

Gel-loading buffer

80% w/v formamide
50 mM Tris-borate (pH 8.3)
1 mM EDTA (pH 8.0)
0.1% w/v xylene cyanol FF
0.1% w/v bromophenol blue

Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

The percentage of polyacrylamide in the solution used to form the gel and the conditions under which electrophoresis is carried out vary according to the size of the oligonucleotides in the reaction mixture. The following table provides useful guidelines:

Length of oligonucleotide	Percent polyacrylamide
12–15 nucleotides	19
25–40 nucleotides	15
40–100 nucleotides	12

Polyacrylamide gels are usually cast in 1 × TBE (89 mM Tris-borate, 2 mM EDTA), and electrophoresis is carried out in the same buffer. For further details of the methods used to cast and handle polyacrylamide gels, see pages 11.23–11.27.

Caution: Acrylamide is a potent neurotoxin and is absorbed through the skin. The effects of acrylamide are cumulative. Wear gloves and a mask when weighing powdered acrylamide and methylenebisacrylamide. Wear gloves when handling solutions containing these chemicals. Although polyacrylamide is considered to be nontoxic, it should be handled with care because of the possibility that it might contain small quantities of unpolymerized acrylamide.

6. Following electrophoresis, disassemble the electrophoresis apparatus, leaving the gel attached to one of the glass plates. **Be careful:** Unincorporated [α-^{32}P]dNTPs may have migrated into the lower buffer reservoir, making it radioactive! Wrap the gel and its backing plate in Saran Wrap. Note the position of the tracking dyes and use a hand-held minimonitor to check the amount of radioactivity in the region of the gel that should contain the oligonucleotide.

Attach a set of adhesive dot labels marked with radioactive ink around the edge of the sample on the Saran Wrap. Cover the dots with Scotch Tape. This prevents contamination of the filmholder or intensifying screen with the radioactive ink.

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Expose the gel to autoradiographic film (see Appendix E). Usually, the amount of radioactivity incorporated into the probe is so great that the time needed to obtain an image on film is no more than a few minutes. Using the radioactive marks on the labels as guides, locate the position of the probe in the gel. Excise the band and recover the radioactive oligonucleotide as described in Chapter 6, pages 6.46–6.47, starting at step 3 and ending at step 8.

Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot (> 2000 cps on a hand-held minimonitor), hot (500–2000 cps on a hand-held minimonitor), and cool (50–500 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

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Conditions for Hybridization of Oligonucleotide Probes

When using oligonucleotides as probes, the aim is to find conditions that are stringent enough to guarantee specificity and sufficiently flexible to allow formation of stable hybrids at an acceptable rate. For DNA molecules more than 200 nucleotides in length, hybridization is usually carried out at 15–25°C below the calculated melting temperature (T_m) of a perfect hybrid. However, as the length of the probe is decreased, the T_m is lowered to the point where it is often impractical to carry out hybridization at $T_m - 25°C$. Typically, therefore, hybridization with synthetic oligonucleotides is carried out under conditions that are only 5–10°C below the T_m. Although such stringent conditions reduce the number of mismatched clones that are detected with short oligonucleotide probes, they have the less desirable consequence of reducing the rate at which perfect hybrids form.

Hybrids formed between DNA molecules more than 200 nucleotides in length are completely stable for all practical purposes. The chances that such a long stretch of double helix will unwind at temperatures 15–25°C below the T_m are extremely small. However, hybrids (even perfect hybrids) formed between short oligonucleotides and their target sequences at 5–10°C below the T_m are far easier to unwind, and hybridization reactions of this type can be regarded as reversible. This has important practical consequences. Whereas hybrids formed between longer DNA molecules are essentially stable under the conditions used for posthybridization washing, hybrids (even perfect hybrids) involving short oligonucleotides are not. Posthybridization washing of such hybrids must therefore be carried out rapidly so that the probe does not dissociate from its target sequence. For this reason, hybridizations with short oligonucleotides should be carried out under stringent conditions (5–10°C below the T_m) using high concentrations (0.1–1.0 pmole/ml) of probe. When only one or a small number of oligonucleotides (<8) are used as probes, the annealing reaction rapidly reaches equilibrium, and hybridization should therefore be terminated after 3 or 4 hours. More complex mixtures, in which the concentration of each oligonucleotide is comparatively low, require hybridization to be carried out for proportionately longer periods. For example, mixtures of 32 or more oligonucleotides should be hybridized for 1–2 days. Posthybridization washing should be carried out for brief periods of time, initially under conditions of low stringency and then under conditions of stringency equal to those used for hybridization.

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CALCULATING MELTING TEMPERATURES FOR PERFECTLY MATCHED HYBRIDS BETWEEN OLIGONUCLEOTIDES AND THEIR TARGET SEQUENCES

When using single oligonucleotides that match the target sequence perfectly, hybridization conditions can easily be derived from the calculated T_m of the hybrid. For oligonucleotides shorter than 18 nucleotides, the T_m of the hybrid can be estimated by multiplying the number of A + T residues in the hybrid by 2°C and the number of G + C residues by 4°C and adding the two numbers (Itakura et al. 1984). However, this method overestimates the T_m of hybrids involving longer oligonucleotides.

A different approach has been taken by E. Fritsch (unpubl.), who found that the equation originally used to calculate the relationship between G + C content, ionic strength of the hybridization solution, and the T_m of long DNA molecules (Bolton and McCarthy 1962):

$$T_m = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%\,G + C) - (600/N),$$

where N = chain length, predicts reasonably well the T_m for oligonucleotides as long as 60–70 nucleotides and as short as 14 nucleotides.

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ESTIMATING THE EFFECTS OF MISMATCHES

Perhaps surprisingly, the classic formula (Bonner et al. 1973) to calculate the effect of mismatches on the stability of long DNA hybrids holds reasonably well for hybrids involving short oligonucleotides: For every 1% of mismatching of bases in a double-stranded DNA, there is a reduction of T_m by 1–1.5°C. However, the precise effect of mismatches depends on the G + C content of the oligonucleotide and, even more critically, on the distribution of mismatched bases in the double-stranded DNA. Mismatches in the middle of the oligonucleotide are far more deleterious than mismatches at the ends. Therefore, the method of estimation given above should only be used as a rough guide until a systematic study of all types of mismatches in a variety of contexts leads to more precise methods of estimation. If appropriate target DNA has been cloned, the effect of mismatches on T_m can be determined empirically (see pages 11.55–11.57).

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It is easy to calculate accurately the T_m of a perfectly matched hybrid formed between a single oligonucleotide and its target sequence. However, when using pools of oligonucleotides whose members have greatly different contents of G + C, it is impossible to estimate a consensus T_m. Because it is not possible to know which member of the pool will match the target sequence perfectly, conditions must be used that allow the oligonucleotide with the lowest content of G + C to hybridize efficiently. Usually, conditions are chosen to be 2°C below the calculated T_m of the most A/T-rich member of the pool (Suggs et al. 1981b). However, the use of such "lowest common denominator" conditions can lead to a number of false positives, because mismatched hybrids formed by oligonucleotides of higher G + C content may be more stable than a perfectly matched hybrid formed by the correct oligonucleotide. In most cases, this problem is not serious, since the number of positive clones obtained by screening cDNA libraries with pools of oligonucleotides is usually quite manageable. It is therefore possible to easily distinguish false positives from true positives by another test (e.g., DNA sequencing or hybridization with a second pool of oligonucleotides corresponding to another segment of amino acid sequence).

In those cases when the number of positives is unacceptably high, it may be worthwhile to consider using hybridization solvents that contain the quaternary alkylammonium salts tetraethylammonium chloride (TEACl) or tetramethylammonium chloride (TMACl) instead of sodium chloride (Melchior and von Hippel 1973; Jacobs et al. 1985, 1988; Wood et al. 1985; Gitschier et al. 1986; Wozney 1989). In these solvents, the T_m of a hybrid is independent of its base composition and dependent primarily on its length. Thus, by choosing a temperature for hybridization appropriate for the lengths of the oligonucleotides in a pool, the effects of potential mismatches can be minimized.

It is important to obtain an accurate estimate of the T_m in TMACl or TEACl before using pools of oligonucleotides to screen cDNA or genomic DNA libraries. Jacobs et al. (1988) measured the T_i (the irreversible melting temperature of the hybrid formed between the probe and its target sequence) as a function of chain length for a number of oligonucleotides of differing G + C content in solvents containing either sodium or tetramethylammonium ions. Hybrids involving oligonucleotides 16 and 19 nucleotides in length melt over a smaller range of temperature in solvents containing TMACl than in solvents containing sodium salts (3°C for TMACl vs. 17°C for SSC when hybridizing 16-mers; 5°C for TMACl vs. 20°C for SSC when hybridizing 19-mers). For 14-mers, the effect is much less dramatic (7°C for TMACl vs. 9°C for SSC). Similar, but less extensive, data are available for solvents containing TEACl (Jacobs et al. 1988).

The optimal temperature for hybridization is usually chosen to be 5°C below the T_i for the given chain length. The recommended hybridization temperature for 17-mers in 3 M TMACl is 48–50°C; for 19-mers, it is 55–57°C; and for 20-mers, it is 58–66°C. Three points are worth emphasizing. First, the T_is of hybrids are uniformly 15–20°C higher in solvents containing TMACl than in solvents containing TEACl. The higher T_i in solvents containing TMACl allows hybridization to be performed at temperatures that

suppress nonspecific adsorption of the probe to solid supports (such as nylon membranes), resulting in lower nonspecific backgrounds. Second, hybridization solvents containing TMACl do not have significant advantages over those containing sodium ions until the length of the oligonucleotide exceeds 16 nucleotides. Finally, the data have been extensively examined for 16-mers, 19-mers, and, in previous studies, for much longer DNA molecules (Melchior and von Hippel 1973). It is currently an untested assumption that the same beneficial effect will be seen for DNA molecules of all intermediate lengths.

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Preparation and Use of Solvents Containing Quaternary Alkylammonium Salts

1. Prepare a 6 M solution of tetramethylammonium chloride (TMACl) or a 3 M solution of tetraethylammonium chloride (TEACl) in H_2O. (TMACl and TEACl are available from Aldrich.)

2. Add activated charcoal to a final concentration of approximately 10% and stir for 20–30 minutes.

3. Allow the charcoal to settle, and then filter the solution of TMACl or TEACl through a Whatman No. 1 paper.

4. Filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size). Store the filtered solution in dark bottles at room temperature.

5. Measure the refractive index of the solution, and calculate the precise concentration of the solution from the equation:

   $C = (n - 1.331)/0.018$

   where C = molar concentration of quaternary alkylammonium salt and n = refractive index.

6. Prehybridize nitrocellulose filters or nylon membranes for 2–6 hours in oligonucleotide prehybridization solution.

Oligonucleotide prehybridization solution

6× SSC (or 6× SSPE)
0.01 M sodium phosphate (pH 6.8)
1 mM EDTA (pH 8.0)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA (see Appendix B)
0.1% nonfat dried milk

7. Prepare the quaternary alkylammonium solution to be used for hybridization:

Quaternary alkylammonium hybridization solution

3.0 M TMACl or 2.4 M TEACl
0.01 M sodium phosphate (pH 6.8)
1 mM EDTA (pH 7.6)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
0.1% nonfat dried milk

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Notes

i. Nitrocellulose filters are not stable when hybridization is carried out for extended periods of time in solvents containing TMACl or TEACl. Nylon membranes are much better suited for this purpose.

ii. Posthybridization washing is usually carried out initially with solutions containing sodium salts (e.g., 6 × SSC) rather than quaternary alkylammonium salts. If additional stringent washes are required, rinse the filters first with quaternary alkylammonium hybridization solution (without DNA or nonfat dried milk) at room temperature and then briefly (5–10 minutes) with the same solution at $T_i - 5°C$.

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HYBRIDIZATION OF GUESSMERS

Perhaps the most critical step in the use of guessmers is the choice of conditions for hybridization. The temperature should be high enough to suppress hybridization of the probe to incorrect sequences but must not be so high as to prevent hybridization to the correct sequence, even though it may be mismatched. Before using an oligonucleotide to screen a library, it is therefore advisable to perform a series of trial experiments in which a series of northern or genomic Southern hybridizations are carried out under different degrees of stringency (Anderson and Kingston 1983; Wood et al. 1984). A set of theoretical curves relating the temperature of the washing solution to the length and homology of the probe is given in Lathe (1985). Using these curves as a guide, determine the optimal conditions for detection of sequences complementary to the probe by hybridizing the oligonucleotide to a series of nitrocellulose filters or nylon membranes at different temperatures. The filters are washed extensively in 6× SSC at room temperature and then briefly (5–10 minutes in 6× SSC) at the temperature used for hybridization. This method, in which both hybridization and washing are carried out under the same conditions of temperature and ionic strength, appears to be more discriminating than the more commonly used procedure of hybridizing under conditions of lower stringency and washing under conditions of higher stringency.

If trial experiments are not possible, attempt to estimate the melting temperature (T_m) as follows:

1. Calculate the minimum G + C content of the oligonucleotide assuming that A or T is present at all positions of ambiguity.
2. Using the following formula, calculate the T_m of a double-stranded DNA with the calculated G + C content:

   $$T_m = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%\ G + C) - (600/N)$$

   where N = chain length.
3. Calculate the maximum amount of possible mismatch assuming that all choices of degenerate codons are incorrect. Subtract 1°C from the calculated T_m for each 1% of mismatch. The resulting number should be the T_m of a maximally mismatched hybrid formed between the probe and its target DNA sequence.

In the absence of information from trial experiments, hybridization and washing should be carried out at 5–10°C below the estimated T_m. Almost certainly, the actual T_m will be higher than that predicted by this worst-case calculation. If the bases used at positions of ambiguity were chosen at random, one out of four should be correct, and approximately half of these would be expected to be G or C. The observed T_m should therefore be significantly higher than that estimated. However, to minimize the risk of missing the clone of interest, it is best to hybridize and wash at several degrees below the T_m estimated as described above. If, under these conditions, the probe hybridizes indiscriminately, repeat the hybridization at a higher temperature or wash under conditions of higher stringency.

Before proceeding to screen an entire cDNA or genomic DNA library, it is advisable to carry out a series of pilot experiments in which the probe is hybridized under different conditions to small aliquots (perhaps 5000–10,000 clones) of the library that is to be screened. The results of these experiments should allow you to choose conditions for large-scale screening that are just stringent enough to eliminate nondiscriminate hybridization of the probe to the vast majority of clones in the library.

Hybridization of guessmers in solvents that contain quaternary alkylammonium salts has not been investigated.

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HYBRIDIZATION OF OLIGONUCLEOTIDES THAT CONTAIN A NEUTRAL BASE AT POSITIONS OF DEGENERACY

Although the conditions for hybridization of probes that contain the neutral base inosine have not been extensively explored, it is possible to make a conservative estimate of the melting temperature (T_m) as follows:

1. Subtract the number of inosine residues from the total number of nucleotides in the probe to give a value S.
2. Calculate the G + C content of S.
3. Estimate the T_m of a perfect hybrid involving S using the equation on page 11.52.
4. Use conditions for hybridization that are 15–20°C below the estimated T_m.

The T_m of hybrids involving oligonucleotides that contain neutral bases can also be estimated empirically as described on pages 11.55–11.57. Hybridization of such oligonucleotides in solvents containing quaternary alkylammonium salts has not been investigated.

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EMPIRICAL DETERMINATION OF MELTING TEMPERATURE

The melting temperature (T_m) of an oligonucleotide hybridized to a target sequence can be determined by the procedure described below. The protocol actually measures the temperature at which dissociation of the double-stranded DNA becomes irreversible (T_i) in nonequilibrium conditions that do not favor rehybridization of the released probe to the target. The optimal temperature for hybridization is then determined on the basis of this value. The procedure requires a cloned target sequence that is complementary (perfectly or imperfectly, depending on the experiment) to the oligonucleotide probe. In most cases, a target sequence is not available from "natural" sources and must be synthesized chemically. The best synthetic target sequences consist of two oligonucleotides that are partially complementary. After annealing, these oligonucleotides form a double-stranded region that contains the target sequence. The sequences of the protruding ends are designed to allow the target DNA to be cloned easily in bacteriophage M13 vectors. Single-stranded DNA of the appropriate orientation prepared from the resulting clones (see Chapter 4) can be used in hybridization experiments as described below. It can also be used as a template for dideoxy-mediated chain-termination sequencing (see Chapter 13) if it is necessary to check that the sequence of the target DNA is correct.

1. Label 1–10 pmoles of the oligonucleotide to be used as a probe by phosphorylation (see pages 11.31–11.32), and remove excess unincorporated [γ-^{32}P]ATP by one of the methods described on pages 11.33–11.39.

2. Using a paper punch, prepare four small circles (diameter 3–4 mm) of a solid support (nitrocellulose filter or nylon membrane) used for hybridization. Arrange the small circles on a piece of Parafilm. Mark two of the filters with a soft-lead pencil.

3. Apply approximately 100 ng of target single-stranded DNA in a volume of 1–3 μl of 2× SSC to each of the marked filters. Apply an equal amount of vector DNA to the unmarked filters. After the fluid has dried, use blunt-ended forceps (e.g., Millipore forceps) to remove the two sets of filters from the Parafilm, and place them between sheets of Whatman 3MM paper. Fix the DNAs to the filters by baking for 1–2 hours at 80°C in a vacuum oven.

   If the target DNA has been cloned into a plasmid, linearize the vector by digestion with a restriction enzyme that does not cleave within the target sequences. Purify the resulting double-stranded DNA by extraction with phenol:chloroform and precipitation with ethanol. Dissolve the DNA in 2× SSC at a concentration of 500 ng/μl. Apply the solution of DNA to the filters prepared as described above, and then, using blunt-ended forceps, transfer the filters to a sheet of 3MM paper saturated with denaturing solution (1.5 M NaCl, 0.5 N NaOH) for 5–10 minutes. Move the filters to a fresh sheet of 3MM paper saturated with neutralizing solution (0.5 M Tris·Cl [pH 7.4], 1.5 M NaCl) for 10 minutes. Transfer the filters to a dry sheet of 3MM paper, and leave them at room temperature until all of the fluid has evaporated. Bake the filters as described above.

   Overbaking can cause the filters to become brittle. In addition, filters that have not been completely neutralized turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

4. Using blunt-ended forceps, transfer all of the filters to a polyethylene tube that contains 2 ml of oligonucleotide prehybridization solution. Seal the tube and incubate, with occasional shaking, at a temperature estimated to be $T_m - 25°C$ for the solvent being used (see Note i). After 2 hours, add radiolabeled oligonucleotide to the prehybridization solution. The final concentration of oligonucleotide should be approximately 1 pmole/ml. Continue incubation at $T_m - 25°C$ for a further 2–4 hours, with occasional shaking.

Oligonucleotide prehybridization solution

6 × SSC (or 6 × SSPE)
0.01 M sodium phosphate (pH 6.8)
1 mM EDTA (pH 8.0)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA (see Appendix B)
0.1% nonfat dried milk

5. Remove the filters from the hybridization solution, and immediately immerse them in 2 × SSC at room temperature. Agitate the fluid continuously. Replace the fluid every 5 minutes until the amount of radioactivity on the filters remains constant (as measured with a hand-held minimonitor).

6. Adjust the temperature of a circulating water bath to $T_m - 25°C$. Dispense 5 ml of 2 × SSC into each of 20 glass test tubes (17 mm × 100 mm). Monitor the temperature of the fluid in one of the tubes with a thermometer. Incubate the tubes in the water bath until the temperature of the 2 × SSC is $T_m - 25°C$. The 2 × SSC in each of these tubes will be used separately for each temperature increase (see steps 7–10).

7. Transfer the filters individually to four empty glass tubes, separating the filters containing the vector and target DNAs, and add 1 ml of 2 × SSC (from one of the tubes prepared in step 6 and prewarmed to $T_m - 25°C$). Place the tubes in the water bath for 5 minutes.

8. Remove the tubes from the bath, transfer the liquid to scintillation vials, and wash the tubes and filters with 1 ml of 2 × SSC at room temperature. Add the wash solutions to the appropriate scintillation vials.

9. Increase the temperature of the water bath by 3°C, and wait for the temperature of the 2 × SSC in the tubes prepared in step 6 to equilibrate.

10. Add 1 ml of 2 × SSC at the higher temperature to each of the four tubes containing the filters. Place the tubes in the water bath for 5 minutes.

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11. Repeat steps 8, 9, and 10 at successively higher temperatures until a temperature of $T_m + 30°C$ is achieved.

12. Place the filters in separate glass tubes (17 mm × 100 mm) containing 1 ml of 2× SSC, and heat them to boiling for 5 minutes to remove any remaining radioactivity. Cool the solutions in ice, and transfer them to scintillation vials. Wash the filters and tubes used for boiling with 1 ml of 2× SSC, and add the washing solutions to the appropriate scintillation vials.

13. Use a scintillation counter to measure the radioactivity (by Cerenkov counting, see Appendix E) in all of the vials. Calculate the proportion of the total radioactivity that has eluted at each temperature (i.e., the sum of radioactivity eluted at all temperatures between $T_m - 25°C$ and the temperature at which a given sample was taken divided by the total radioactivity eluted from the filters at all temperatures up to and including 100°C).

    If the experiment has worked well, very little radioactivity should be associated with the filters containing vector DNA alone. Furthermore, this radioactivity should be completely released from the filters at temperatures much lower than the estimated T_m. On the other hand, considerable radioactivity should be associated with the filters containing the target DNA; the elution of this radioactivity should show a sharp temperature dependence. Very little radioactivity should be released from the filters until a critical temperature is reached, and then approximately 90% of the radioactivity should be released during the succeeding 6–9°C rise in temperature. The temperature at which 50% of the radioactivity has eluted from the filters containing the target sequences is defined as the T_i of the hybrid between the probe and its target sequence.

Notes

i. Although the above protocol calls for the use of sodium salts in the solvent used for hybridization, other solutes such as tetramethylammonium chloride or tetraethylammonium chloride can be substituted if desired to determine the T_i in these solvents.

ii. This method can easily be adapted to study the behavior of hybrids formed between probes and target sequences that do not match each other perfectly (Jacobs et al. 1988).

iii. Before synthesizing the probe, check for potential homology and/or complementarity between its sequence and the sequence of the vector used to propagate the target. Most of the commercially available programs to analyze DNA can be used to search commonly used vectors for sequences that match the sequence of the probe closely enough to cause problems during hybridization.

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Screening Expression Libraries with Antibodies and Oligonucleotides

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Large numbers of cDNAs have been cloned by screening libraries with synthetic single-stranded oligonucleotides corresponding to sequences of amino acids obtained by analysis of the purified target protein. However, many other genes are expressed either in mammalian tissues that are difficult to obtain or at such low levels that it is difficult to purify sufficient protein to obtain sequence information. Several methods have been devised to identify individual clones that synthesize such proteins in recombinant libraries constructed in expression vectors. In every case, these methods involve screening the library with a ligand that binds specifically to the protein of interest. Such ligands fall into three classes: (1) small compounds or proteins that are known to interact with the protein of interest in vivo, (2) immunoglobulins directed against antigenic epitopes displayed by the protein of interest, and (3) short sequences of DNA recognized by proteins, such as *trans*-acting factors involved in transcriptional control of eukaryotic genes.

Small ligands are used to exploit the special biochemical properties of the target protein. For example, ^{125}I-labeled calmodulin has been used to identify clones in a murine brain cDNA library that bind to the radiolabeled ligand in the presence of calcium (Sikela and Hahn 1987). Similarly, ^{125}I-labeled α-bungarotoxin has been shown to bind to a fusion protein containing part of the α subunit of the acetylcholine receptor (Barkas et al. 1987), and recombinants expressing the regulatory subunit of a cAMP-dependent protein kinase have been successfully identified by screening a cDNA library constructed in bacteriophage λgt11 with ^{32}P-labeled cAMP as a ligand (Lacombe et al. 1987). Clearly, this method can be used only when a specific ligand is available that binds with high affinity to the protein of interest (e.g., the K_d of the α-bungarotoxin to renatured α subunit of acetylcholine receptor complex is 0.3 nM; Tzartos and Changeux 1984). The chances of success are increased if the ligand is small (e.g., a metal ion or a low-molecular-weight agonist) and binds to a small tract of contiguous amino acids (as is the case with α-bungarotoxin; Wilson et al. 1985), rather than to a complex structure formed by the interaction of several distant segments of the polypeptide chain. However, even if these conditions are fulfilled, the strength of the signal may be comparatively weak: Only a small proportion of the foreign protein synthesized in bacteria may be folded into a form competent for binding. The affinity of a malfolded or unmodified protein for its ligand may also be much lower than that of the homologous protein synthesized in eukaryotic cells (Barkas et al. 1987). These factors probably account for many of the difficulties encountered in using more complex ligands to screen expression libraries.

Immunological methods can, in principle, be used to detect any protein for which a specific antibody is available. Although antibodies are complex ligands, they have the advantage of binding specifically, and with high affinity, to only a small region (epitope) of the antigen. Although some epitopes are complicated topological structures formed by the folding of noncontiguous regions of one or more polypeptide chains, others consist of tracts of adjacent amino acids from a single polypeptide chain (Geysen et al. 1987). Such localized epitopes can be formed in foreign proteins expressed in bacteria even though the protein as a whole may be malfolded and nonfunctional. In addition, fusion proteins or fragments of protein can form simple epitopes and will therefore retain at least a part of their immunological

reactivity. Because of their ability to react with cognate epitopes when the surrounding protein is malfolded or denatured, antibodies are the reagents of choice for primary screening of expression libraries when no nucleic acid probes are available for the gene of interest.

Recently, synthetic *double-stranded* oligonucleotides corresponding to sequences derived from the upstream regulatory elements of mammalian genes have been used successfully to screen cDNA libraries constructed in prokaryotic expression vectors (such as bacteriophage λgt11) for clones expressing proteins that bind specifically to the oligonucleotide probes (Singh et al. 1988; Vinson et al. 1988). The DNA-binding domains of these proteins include such structural motifs as zinc fingers (Miller et al. 1985; for review, see Evans and Hollenberg 1988), leucine zippers (Landschulz et al. 1988a; O'Shea et al. 1989), helix-turn-helix (for review, see Pabo and Sauer 1984; Schleif 1988). Previously, cDNAs encoding transcriptional regulatory proteins were cloned by (1) screening of expression libraries by nucleic acid hybridization with *single-stranded* oligonucleotides corresponding to sequences of amino acids found in the purified protein (Walter et al. 1985; Kadonaga et al. 1987; Bodner et al. 1988) or (2) screening with antibodies raised against the purified protein (Walter et al. 1985; Weinberger et al. 1985; Landschulz et al. 1988b). These conventional approaches were made possible by the development of methods to purify specific DNA-binding proteins by affinity chromatography. In these methods, proteins are partially purified using conventional chromatographic resins and then passed through a column containing an immobilized fragment of DNA carrying several copies of a specific DNA-binding site (Chodosh et al. 1986; Kadonaga and Tjian 1986; Rosenfeld and Kelly 1986). The protein of interest binds to the column and can be specifically eluted in buffers of high ionic strength. It can then be used to raise antibodies or can be partially sequenced to generate single-stranded oligonucleotide probes.

Although an enrichment of several thousandfold can be achieved by DNA affinity chromatography (Kadonaga et al. 1987; Landschulz et al. 1988b), the method is time-consuming and expensive, especially if the protein of interest is rare. For example, Vinson et al. (1988) used approximately 1×10^{11} nuclei to purify 3 μg of a 14-kD DNA-binding protein. The ability to screen expression libraries by ligand binding with synthetic double-stranded oligonucleotides greatly simplifies the identification of cDNAs coding for nonabundant proteins that bind specifically to DNA. However, it is important to note that this screening method does not entirely obviate the more conventional approaches used to isolate this class of cDNAs; DNA-binding proteins that are composed of multiple subunits or require in vivo modification for binding activity will not be detected by expression screening.

In this chapter, we first summarize important factors that affect the outcome of screening expression libraries with antibodies and then describe in detail the procedures that are in common use. In addition, we provide a detailed protocol for screening cDNA libraries constructed in expression vectors for specific DNA-binding proteins using oligonucleotides containing recognition sequences.

Constructing Expression Libraries in Plasmid and Bacteriophage λ Vectors

The Relative Advantages of Plasmid and Bacteriophage λ Expression Vectors

Prokaryotic expression libraries can be constructed in either plasmid or bacteriophage λ vectors that carry a strong, regulatable prokaryotic promoter and a ribosome-binding site. A few vectors are available that express only the sequences of the foreign protein (see Chapter 17). More commonly, however, the foreign DNA sequences are cloned into a restriction site that lies within the coding sequences of a prokaryotic gene (or fragment of a gene) located immediately downstream from the promoter/ribosome-binding site. In most vectors, this gene is *Escherichia coli lacZ*, which codes for β-galactosidase. If the foreign sequences are inserted in the correct orientation and reading frame, a fusion protein is produced consisting of β-galactosidase sequences linked to polypeptide sequences encoded by the cloned DNA. In several cases, fusion of the foreign sequences to a stable *E. coli* protein such as β-galactosidase has been shown to enhance the stability of the fusion protein (Küpper et al. 1981; Stanley 1983). The fusion protein may retain or lose β-galactosidase activity or the ability to take part in α-complementation (see Chapter 1), depending on the nature of the foreign sequences and the site at which they are inserted. Colonies (in the case of plasmids) or bacteriophage plaques (in the case of bacteriophage λ) are then screened for the presence of material that reacts with antisera specific for the protein of interest. Clones encoding a wide variety of proteins have been isolated in this way from both types of libraries. For example, antibodies have been used to identify recombinant clones in a number of different plasmid vectors (Broome and Gilbert 1978; Erlich et al. 1978; Kemp and Cowman 1981; Koenen et al. 1982; Meyer et al. 1982; Rüther et al. 1982; Helfman et al. 1983). More recently, following the widespread adoption of bacteriophage λ as a vector for the construction of cDNA libraries (Young and Davis 1983a,b), immunological screening of recombinant clones has come into generalized use. More than 200 genes have now been isolated using this technique, including rat fibronectin (Schwarzbauer et al. 1983), cat brain glutamate decarboxylase (Kaufman et al. 1986), yeast clathrin (Payne and Schekman 1985), rat thyrotropin-releasing hormone (Lechan et al. 1986), firefly luciferase (de Wet et al. 1985), and *Neurospora* plasma membrane ATPase (Hager et al. 1986).

Although immunological screening of expression libraries constructed in bacteriophage λ vectors has been highly successful, it is important to understand that screening of plasmid libraries may have advantages in certain circumstances. Expression libraries constructed in bacteriophage λ and plasmid vectors differ from one another in one fundamental respect. Libraries constructed in bacteriophage λ vectors are plated on a bacterial lawn, which is progressively lysed during the next few hours as a natural consequence of bacteriophage growth. The resulting debris is transferred to

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nitrocellulose filters and screened for the presence of immunoreactive material. In contrast, plasmid libraries are maintained as transformed bacterial colonies that must be lysed in situ on nitrocellulose filters before intracellular fusion proteins can be screened with antibodies. With plasmid libraries, it may be possible for the investigator to select conditions for lysis that may better preserve the native structure of the protein of interest. Plasmid vectors may therefore be superior to bacteriophage λ vectors when complex ligands that bind to complicated topological structures are used for screening.

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Genomic DNA and cDNA Expression Libraries

Two types of expression libraries can be constructed in plasmid and bacteriophage λ expression vectors: cDNA and genomic DNA libraries. For prokaryotic organisms, whose genomes contain few introns, there is little to be gained by constructing cDNA libraries in these vectors. Instead, randomly sheared genomic DNA can be used to create a library (for details, see Chapter 9) in which all coding sequences are proportionally represented (see, e.g., Young et al. 1985). The optimal average length of the sheared fragments of DNA used to construct libraries of this kind depends on the size of the gene of interest: the smaller the gene, the smaller the genomic DNA fragments. Ideally, the size of the fragments should be approximately one half the predicted size of the gene. This reduces the chance that the coding sequences of the gene of interest will be preceded by upstream termination sequences.

A similar strategy can be used with eukaryotic organisms such as yeasts, whose genomes are comparatively small and consist predominantly of coding sequences. The probability of creating a recombinant containing the sequences of the gene of interest in the correct reading frame and orientation for expression is:

$$P = \frac{\text{size of the coding region of the gene of interest (in kb)}}{\text{size of genome (in kb)} \times 6}$$

The factor of 6 takes into account the fact that a given DNA fragment can be inserted into the vector in two possible orientations and that only one of the three potential reading frames is correct for expression. The proportion of clones expressing the particular fusion protein is then $1{:}1/P$. For example, in a genomic DNA expression library, the proportion of recombinants capable of expressing a fusion protein encoded by a gene whose coding region is 1.5 kb in length is predicted to be:

	Genome size (kb)	Proportion
E. coli	4.0×10^3	$1{:}1.6 \times 10^4$
Yeast	1.4×10^4	$1{:}5.6 \times 10^4$
Drosophila	1.8×10^5	$1{:}7.2 \times 10^5$

In theory, these frequencies will be attained only if the cloned genomic DNA fragments are considerably smaller ($\leq 50\%$) than the size of the coding sequence of interest. In practice, however, the frequency of positive clones is somewhat higher than expected. Although the reasons for this are not understood, a possible clue comes from the observation that a high proportion of clones isolated from genomic DNA expression libraries by immunological screening do not synthesize fusion proteins (Goto and Wang 1984; Özkaynak et al. 1984; Snyder et al. 1986). Because expression of these cloned genes is dependent on transcription from the *lacZ* promoter, it seems likely that the clones carry out-of-frame insertions and that translation of the foreign sequences is initiated at an internal methionine codon. More rarely, the foreign sequences may carry signals that can direct transcription and translation in *E. coli*.

As the size of eukaryotic genomes increases, the fraction that is occupied by coding sequences shrinks dramatically and the average length of individual exons decreases to approximately 200 bp (Gō 1983). The idea of screening genomic DNA expression libraries then becomes much less attractive. In the case of the mammalian genome (3×10^9 bp), reconstruction experiments suggest that it would be necessary to screen a library of at least 10^7 clones

(average size 200–300 bp) to detect a recombinant expressing a particular epitope (Rüther et al. 1982). Even then, only epitopes encoded by DNA sequences contained within a single exon would be detected. The use of cDNA libraries solves these problems by consolidating the coding sequences of individual genes into single clones and eliminating the nonproductive screening of clones that consist of intron and repetitive sequences.

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Expression Libraries Constructed in Plasmids

Most cDNA expression libraries constructed in plasmid expression vectors use plasmids of the pUC, pUR, or pEX series (see Chapters 1 and 17). In the pUC and pUR vectors, the cDNA is inserted into polycloning sites that lie within the amino-terminal region (pUC) or the carboxy-terminal region (pUR) of the *E. coli* β-galactosidase gene. Although libraries constructed in both pUR and pUC vectors have been screened successfully with antibodies (Rüther et al. 1982; Helfman et al. 1983), pUR vectors have some advantages. First, because the polycloning site is available in all three translational reading frames, any open reading frame of a sequenced fragment of DNA can be easily cloned for expression. Second, recombinants carrying an insertion at the 3′ terminus of the *lacZ* gene direct the synthesis of more stable fusion proteins than those with insertions at the 5′ terminus (Stanley 1983). In at least some cases, these fusion proteins retain β-galactosidase activity (Rüther and Müller-Hill 1983), which can serve as a useful marker during protein purification. Expression of fusion proteins in both pUC and pUR plasmids is controlled by the upstream *lac* promoter and is therefore repressed in strains of bacteria that overproduce *lac* repressor (I^q strains). Continual repression is important to prevent constitutive expression of foreign proteins, which are frequently toxic to *E. coli* (Amann et al. 1983; Gething and Sambrook 1983). Plasmids that code for deleterious proteins can therefore be amplified under conditions that allow only minimal expression of the fusion protein. Maximal synthesis of the fusion protein is transiently induced by exposing the culture to the nonmetabolized analog of lactose—isopropylthio-β-D-galactoside (IPTG)—before immunological screening is undertaken. (Recently, several expression plasmids have been described that carry their own copy of the $lacI^q$ gene [Bagdasarian et al. 1983; Masui et al. 1984; Stark 1987]. Expression of sequences cloned in these plasmids is therefore tightly regulated, regardless of the strain of bacteria used.)

The pEX family of plasmids also contains polycloning sites in all three reading frames at the 3′ terminus of a fusion gene composed of bacteriophage λ *cro* sequences and *E. coli lacZ* sequences (Stanley and Luzio 1984). Expression of *cro*–β-galactosidase fusion proteins is controlled by the bacteriophage λ p_R promoter, which can be repressed during growth and amplification of the plasmid by the bacteriophage λ *c*I gene product. The pEX vectors are normally grown at 30°C in a bacterial strain carrying a defective bacteriophage λ prophage that synthesizes a temperature-sensitive *c*I gene product. Transient expression of the fusion protein is induced by raising the temperature of incubation to 42°C before immunological screening.

Although quantitative comparisons have not been carried out, it is clear that each of these three families of plasmids can efficiently direct the synthesis of large quantities of fusion proteins (up to 40% of the total extractable cellular protein). Within any one system, smaller fusion proteins are expressed to higher levels than larger fusion proteins and are degraded less rapidly by cellular proteases, perhaps because they more readily form precipitates (known as inclusion bodies) within the cell (Stanley and Luzio 1984).

Despite their efficiency, expression libraries constructed in plasmids suffer from a major disadvantage: the labor involved in generating the replicas of

the library that are used for immunological screening. Usually, master copies of plasmid libraries are grown on nitrocellulose filters, and several replicas are then generated by transferring the colonies in situ to fresh nitrocellulose filters (Hanahan and Meselson 1980) (see page 12.21). This is an extremely laborious process that can be repeated only a few times before the colonies on the master filter become smeared beyond recognition. Colonies growing on the replica filters are lysed and screened with antiserum specific for the gene product of interest. Clones that react with the antibody are recovered from the master filter and analyzed further. Although these procedures are easy to accomplish when only a few clones are to be screened, they are logistically demanding when large libraries consisting of 10^6 or more colonies are to be screened. For most purposes, therefore, bacteriophage λ is the vector of choice for immunological screening of large libraries.

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Expression Libraries Constructed in Bacteriophage λ

The vast majority of cDNA libraries constructed in bacteriophage λ expression vectors use λgt11 (or its close relatives λgt18–23), λZAP, or λORF8. These bacteriophages carry a copy of the *E. coli lacZ* gene, with a cloning site located 53 bp upstream of the translational termination codon. cDNAs inserted in the correct orientation and reading frame are expressed to yield fusion proteins whose amino termini consist of β-galactosidase sequences and whose carboxyl termini consist of foreign sequences. Some of these foreign sequences display antigenic epitopes that can be detected using specific antibodies.

Bacteriophage λ vectors λgt11 and its derivatives λgt18–23 code for a temperature-sensitive repressor (c*Its*857) and carry an amber mutation in the *S* (lysis) gene. At temperatures that inactivate the repressor, such strains form plaques on strains of *E. coli* (such as Y1090*hsdR*) that carry the amber suppressor (*supF*). Strain Y1090*hsdR* displays additional properties that are useful in immunological screening: It is deficient in the *lon* protease (for review, see Mount 1980), and it carries a plasmid (pMC9) that codes for the *lac* repressor. For further details of the properties of λgt11, λgt18–23, λZAP, and λORF8, see Chapters 2 and 8.

For immunological screening, libraries constructed in bacteriophage λgt11 or its derivatives are plated on strain Y1090*hsdR* at 42°C (to prevent lysogenization) in the absence of isopropylthio-β-D-galactoside (IPTG) (to ensure that no fusion proteins toxic to the host are synthesized until plaque formation is well under way). After 3–4 hours, the plates are moved to 37°C (to stabilize any fusion proteins that are temperature-sensitive) and filters impregnated with IPTG are laid on top of the developing plaques (to collect an imprint of the induced fusion proteins). After incubation for a further 3–4 hours, the filters are removed and probed with antibody as described in step 12, page 12.19. The plates are stored at 4°C until the results of immunological screening are available. Positive plaques are recovered and analyzed further.

Note: λgt11 and its derivatives express a temperature-sensitive repressor and can form lysogens efficiently at 32°C. In the original description of λgt11 (Young and Davis 1983a), cDNA libraries constructed in λgt11 were screened as bacterial lysogens. The colonies were induced by heating and screened for subsequent production of foreign antigens. Propagation and screening of expression libraries as lysogens appeared to offer two potential advantages: λgt11 genomes inherited as part of the host-cell chromosome would perhaps be more stable genetically than genomes propagated by lytic infection, and the repression of synthesis of potentially toxic fusion proteins might be more complete in cells containing only one integrated copy of the recombinant genome than in lytically infected cells that rapidly accumulate many hundreds of copies of the fusion gene. Despite these apparent advantages, libraries constructed in λgt11 are no longer propagated as lysogens nor screened after induction because some recombinants are unable to form stable lysogens (Huynh et al. 1985). Furthermore, the signal-to-noise ratio is much higher when plaques, rather than colonies, of induced lysogens are screened with immunological probes.

Using Antibodies in Immunological Screening

Choosing the Antibody

Ideally, an antibody used for screening of expression libraries should be polyclonal, absolutely specific for conformation-independent epitopes that are displayed on both native and denatured forms of the protein, of high titer, and of the IgG class. This combination of properties is rarely found in a single antiserum, and it is often necessary to screen expression libraries with preparations of antibodies that are far from ideal. To determine whether the available batches of antisera are suitable as probes, serial dilutions of several independent antisera should be tested for the following properties:

- *Reactivity with the protein of interest on a western blot.* Denatured proteins transferred from an SDS-polyacrylamide gel are likely to display many of the same epitopes as the cognate fusion protein synthesized in bacteria. The foreign polypeptide sequences of most fusion proteins are unlikely to fold into completely native structures and, after transfer to a nitrocellulose filter, may undergo further denaturation. Antisera that fail to detect the protein of interest on a western blot are probably specific for epitopes displayed only on the native protein and therefore may be unsuitable as probes for expression libraries.

- *Specific immunoprecipitation of a polypeptide of the predicted size from in vitro translation reactions primed with the same mRNA used to construct the expression library.* An antiserum reacting strongly in this test must be directed primarily against epitopes that are displayed on unmodified, and probably unfolded, proteins. Clearly, antisera that recognize oligosaccharides or other modifying groups found on eukaryotic proteins are of no value for screening prokaryotic expression libraries.

- *Production of weak or undetectable signals when reacted with bacteria expressing the sequences of the vector alone.* Antisera should be tested by screening two nitrocellulose filters, one containing imprints of nonrecombinant plaques or colonies formed by the vector alone and the other imprints of a small fraction (e.g., 1–5000 plaques or colonies) of the expression library that is to be screened. A series of dilutions containing known amounts of the native and denatured (boiled) antigen (20 pg to 100 ng) of interest are spotted onto a separate filter. The three filters are then cut into sections, which are screened as described below with different dilutions of antiserum. The aim is to find a dilution that displays a high level of specific reactivity with the antigen but does not react nonspecifically with prokaryotic proteins. Typically, the range of dilutions used in this test is 1:200–1:5000 for polyclonal antisera, IgG fractions, ascitic fluids, and affinity-purified antisera and 1:10–1:100 for supernatant fluids from cloned hybridomas grown in vitro. Ideally, the antibody should be capable of detecting as little as 50–100 pg of antigen in an area the size of a small bacterial colony. The amount of antigen present in colonies and plaques

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expressing fusion proteins varies widely depending on the toxicity of the fusion protein, its rate of degradation, and its physical state. However, an antiserum capable of detecting as little as 50–100 pg of denatured antigen should allow all but the most labile of fusion proteins to be recognized.

Both polyclonal antisera and monoclonal antibodies can meet the above criteria, and both have been used successfully to identify recombinants that express cloned sequences of interest. Each has different advantages. Polyclonal antisera are usually able to react with several different epitopes, which may be encoded by different regions of the gene of interest. Recombinants carrying fragments of this gene may therefore be detected by polyclonal antisera. In contrast, monoclonal antibodies, which by definition are able to react with only one epitope, may detect only a particular subset of the recombinants that express sequences of the gene of interest. However, monoclonal antibodies generally show much lower levels of background reactivity with *E. coli* components than do polyclonal antisera. The best reagents for immunological screening are therefore pools of monoclonal antibodies directed against different epitopes on the protein of interest or high-titer polyclonal antibodies that do not react with host components.

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Purification of Antisera

When the antisera to be used for screening react with only one protein of the expected molecular weight on a western blot, further antigen-specific purification is not necessary. However, if the antibodies that react with the antigen of interest are of the IgG class, they may be easily purified and concentrated by affinity chromatography on protein A–Sepharose columns (Goudswaard et al. 1978). Preparations of IgG used for immunological screening usually give lower backgrounds than whole sera.

Antisera that react with several different proteins usually have been raised against impure antigen and are therefore likely to react with recombinant clones other than those specifying the protein of interest. In this case, the antibody should be purified by affinity chromatography on columns of the immobilized antigen. Note that affinity purification must be carried out with an independent preparation of antigen that is free of the contaminants present in the material used for immunization. If such a preparation is impossible to obtain, there are two options. First, the library may be screened with the available polyclonal antisera; immunoreactive clones must then be analyzed by a test that can distinguish unambiguously between false and true positives. Alternatively, impure preparations of antigen may be used to generate clonal lines of hybridomas that secrete monoclonal antibodies specific for the protein of interest.

Components in polyclonal antisera or preparations of IgG that react with antigens produced by *E. coli* may be removed by immunoabsorption, for example, by incubating the diluted antiserum with nitrocellulose filters coated with lysed bacterial colonies or the debris of nonrecombinant plaques (pseudoscreening; see page 12.25). This process can be repeated several times, until all of the anti-*E. coli* antibodies have been removed. Alternatively, the diluted antiserum can be mixed with an extract of *E. coli* made by freeze-thawing and sonication. The absorbed diluted antiserum can then be used to probe expression libraries without further treatment. The most effective procedure is to expose undiluted antiserum or purified IgG to lysates of *E. coli* that have been immobilized on cyanogen-bromide-activated Sepharose 4B as described by de Wet et al. (1984) (see page 12.27).

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Methods Used to Detect Antibodies Bound to Proteins Expressed in E. coli

Among the reagents that have been used to detect antibody bound to its antigen on nitrocellulose filters are:

- ^{125}I-labeled antibodies reactive with species-specific determinants on the primary antibodies (Helfman et al. 1983).
- Protein A of *Staphylococcus aureus* radiolabeled with ^{125}I (Kemp and Cowman 1981; Johnson et al. 1985).
- Second antibodies conjugated directly to horseradish peroxidase (HRP) (de Wet et al. 1984). Alternatively, HRP coupled to avidin may be used to detect a second antibody coupled to biotin (Young et al. 1985; French et al. 1986).
- Second antibodies conjugated to alkaline phosphatase (AP) (Mierendorf et al. 1987).
- Radiolabeled ligand, which binds to divalent antibody that is attached monovalently to antigen on the nitrocellulose filter (Chao et al. 1989). This method can be used only in those rare circumstances when pure ligand is available in quantity.

Although a systematic comparison of these reagents has not been published, enzymatic methods of detection are generally more sensitive and give lower backgrounds than radiochemical methods. In addition, enzymatic methods produce signals directly on the nitrocellulose filter (rather than on a sheet of X-ray film), so that positive plaques can be located more accurately.

Antibodies coupled to either HRP or AP that react with species-specific determinants on the primary antibodies are available commercially, and both have been used successfully to isolate cDNA clones from expression libraries constructed in bacteriophage λ vectors. Both enzymes catalyze the formation of an insoluble, colored precipitate on the surface of a nitrocellulose filter at the site of an antigen-antibody complex (Towbin et al. 1979; Hawkes et al. 1982; Blake et al. 1984; Knecht and Dimond 1984). AP, however, offers some potential advantages: The enzyme remains active for several hours, during which the end product of the reaction—a dark-blue precipitate of diformazan (McGadey 1970)—continues to accumulate and intensify in color. In contrast, although HRP has a higher turnover number, it is inactivated within a short period of time by its substrate, peroxide. This may account for the lower sensitivity of HRP-conjugated antibodies in detecting antigen-antibody complexes on the surfaces of nitrocellulose filters (Mierendorf et al. 1987). Furthermore, some protocols that use HRP-conjugated antibodies require the use of a carcinogenic chromogen, *o*-dianisidine, which requires special handling and disposal.

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Validation of Clones Isolated by Immunological Screening

The identification of immunoreactive clones from expression libraries cannot be taken as proof that the cDNA of interest has been isolated. The specificity of antibodies is never absolute and the epitope(s) recognized on the protein is usually undefined. Further tests are therefore required to validate the identity of the initial isolates. In decreasing order of stringency, these tests include:

- *Demonstration that the protein encoded by the cloned cDNA displays the correct biological or enzymatic activity.* This test usually requires the isolation of full-length cDNA clones and expression in prokaryotic or eukaryotic vectors that do not generate fusion proteins. In only a few cases has it been possible to demonstrate the appropriate enzymatic activity in fusion proteins synthesized in bacteriophage λgt11 vectors (e.g., firefly luciferase [de Wet et al. 1985] and cat brain glutamate decarboxylase [Kaufman et al. 1986]).

- *Comparison of the amino acid sequence of the protein with the amino acid sequence deduced from the sequence of the cloned cDNA.* Unfortunately, this test can rarely be applied, since there is usually no amino acid sequence available for proteins whose genes are cloned by immunological screening.

- *Demonstration that the fusion protein expressed by the clone can block the interaction between the purified protein of interest and a specific antibody.* For example, the fusion protein could protect an enzyme from inactivation by an antibody. Alternatively, the fusion protein can be used as a ligand in affinity purification of an antibody (Olmsted 1981; Smith and Fisher 1984; Earnshaw and Rothfield 1985). The affinity-purified antibody is then used as a probe in western blots of purified antigen. Finally, the fusion protein may be used as an immunogen to raise antibodies that can be tested for their ability to bind to, or inhibit the activity of, the protein of interest.

These are by no means the only tests that may be used to validate clones isolated by immunological screening. However, whichever method is used, it is essential to devise independent tests that do not rely on the same immunological reagents used to identify the clone in the first place.

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Immunological Screening of Expression Libraries

SCREENING EXPRESSION LIBRARIES CONSTRUCTED IN BACTERIOPHAGE λ VECTORS

1. Using a single colony of the appropriate strain of *E. coli* as inoculum, prepare a plating culture as described in Chapter 2.

   E. coli strain Y1090*hsdR*, which is commonly used as the host for immunological screening of expression libraries constructed in λgt11, carries a plasmid (pMC9) that codes for the *lac* repressor and prevents synthesis of potentially toxic fusion proteins from the β-galactosidase promoter. This plasmid also carries a selectable marker (*amp*^{r}). To ensure against loss of the plasmid, *E. coli* strain Y1090*hsdR* should be grown in media containing 50 μg/ml ampicillin.

   E. coli strains BB4 and XL1-Blue, which are used for immunological screening of libraries constructed in λZAP, carry a *lacI*^{q} gene and a *tet*^{r} marker on an F′ factor. These strains should therefore be grown in media containing 12.5 μg/ml tetracycline.

2. Calculate the number of plates that will be required to screen the library, assuming 2×10^4 plaques per 90-mm plate or 5×10^4 plaques per 150-mm plate. Arrange a set of sterile tubes (13 mm × 100 mm) in a rack; use a fresh tube for each plate that is to be infected. In each tube, mix 0.1 ml of the plating bacteria with 0.1 ml of SM containing 3×10^4 pfu (90-mm plates) or 10^5 pfu (150-mm plates) of the bacteriophage λ expression library. Incubate the infected bacteria for 20 minutes at 37°C.

   Immunological screening works best when the density of plaques is low. The color produced by the chromogenic reaction is most intense at the expanding edge of the plaque. Well-isolated immunopositive plaques therefore display a characteristic ringlike pattern. When plaques crowd against one another, their morphology becomes fragmented and the intensity of color at the common border between adjacent plaques is greatly reduced. However, immunopositive plaques can still be recognized as long as some portions of their borders do not make contact with the borders of neighboring plaques. When the density exceeds approximately 500 plaques/cm^2, the borders between neighboring plaques disappear and antigen-positive plaques become increasingly difficult to identify. The number of plaques per plate given above should therefore be regarded as the upper limit for immunological screening.

3. Add to each tube 2.5 ml (90-mm plate) or 7.5 ml (150-mm plate) of molten top agarose, and immediately pour the mixture onto an LB agar plate. The plates must be dry; otherwise, the top agarose will peel off when the nitrocellulose filter is removed. Usually, 2-day-old plates that have been dried for an additional 1–2 hours at 37°C with the lids slightly open work well. Incubate the infected plates for 3.5 hours at 42°C.

4. Number nitrocellulose filters with a soft-lead pencil or a ballpoint pen. The filters should be handled with gloved hands, since skin oils prevent the transfer of proteins. Soak the filters in a solution of isopropylthio-β-D-galactoside (IPTG) (10 mM in distilled water) for a few minutes. Using

blunt-ended forceps (e.g., Millipore forceps), remove the filters from the solution, and allow them to dry at room temperature on a pad of Kimwipes.

Nitrocellulose filters that are free of Triton X-100 (Millipore HATF or equivalent) give the best results.

5. Remove the plates from the incubator, and quickly overlay them with the IPTG-impregnated nitrocellulose filters. Do not move the filter once contact is made with the plate. The easiest way to place the filter on the plate without trapping air bubbles is to hold it by its edges, bending it slightly so that the middle of the filter makes contact with the center of the plate. Wetting action then pulls the filter onto the plate.

Do not allow the temperature of the plates to drop below 37°C; otherwise, the growth of the bacteriophages will be retarded.

6. Incubate the plates for at least 4 hours at 37°C.

If the library is not to be screened in duplicate, the original filter can be left on the plate for 10–12 hours. If duplicate filters are required, remove the first filter after 4 hours and replace it with a second numbered filter that has been impregnated with IPTG (see notes to step 9). Then incubate the plate for a further 4–6 hours at 37°C before the second filter is removed.

During primary screening of a library, it is often difficult to distinguish immunoreactive plaques from false positives. The frequency with which false positives appear depends on several factors, including the care taken in handling the filters, the characteristics of the particular antiserum, and the method used to detect antigen-antibody complexes. In general, chromogenic methods of detection yield fewer false positives than radiochemical methods. Even under the best conditions, however, false positives arise at a frequency of approximately one per plate. To avoid the labor and expense of screening large numbers of plaques for a second time, we recommend that primary screening be carried out in duplicate.

7. Remove the lids from the plates and continue the incubation for a further 20 minutes at 37°C. This helps to prevent the top agarose from sticking to the filter rather than to the plate.

8. Move the plates in small batches to room temperature. Mark each filter in at least three asymmetric locations by stabbing through it and into the agar underneath with an 18-gauge needle attached to a syringe containing waterproof black ink.

9. Using blunt-ended forceps, peel the filters off the plates and immediately immerse them in a large volume of TNT. Rinse away any small remnants of agarose by gently agitating the filters in the buffer. Gently agitate the TNT to prevent the filters from sticking to one another.

TNT

10 mM Tris · Cl (pH 8.0)
150 mM NaCl
0.05% Tween 20

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Wrap the plates in Saran Wrap, and store them at 4°C until the results of the immunological screening are available.

If large areas of the top agarose stick to the nitrocellulose filters, chill the plates for 30 minutes at 4°C or 5 minutes at −20°C before peeling off the filters.

To prepare duplicate filters:

a. Remove the lids from the plates 30 minutes before removing the first filter.
b. Chill the plates for 30 minutes at 4°C or 5 minutes at −20°C.
c. Remove the filters and immediately immerse them in TNT.
d. Overlay each of the plates with a second filter impregnated with IPTG.
e. Incubate the plates for a further 4–6 hours at 37°C.
f. Meanwhile, process the first set of filters as described in the following steps.
g. Remove the second set of filters 4–6 hours later, immerse them in TNT, and process them as described in the following steps.

Important: Do not allow the filters to dry during any of the subsequent steps. Antibodies bound nonspecifically and reversibly to wet filters become permanently attached if the filters dry out. It is also essential that the filters do not stick to one another when they are immersed in the various buffers and antibody solutions. This problem can be minimized by dividing the filters into small batches (five filters per batch) and using a separate large petri dish or crystallizing dish for each batch. The petri dishes can be stacked on top of one another on a slowly rotating platform shaker.

10. When all of the filters have been removed and rinsed, transfer them one at a time to a fresh batch of TNT. When all of the filters have been transferred, agitate the buffer gently for a further 30 minutes at room temperature.

If necessary, the filters may be removed from the buffer at this stage, wrapped in Saran Wrap, and stored for up to 24 hours at 4°C.

11. Using blunt-ended forceps, transfer the filters individually to glass trays or petri dishes containing blocking buffer (7.5 ml for each 82-mm filter; 15 ml for each 138-mm filter). When all of the filters have been submerged, agitate the buffer slowly on a rotary platform for 30 minutes at room temperature.

Blocking buffer

20% fetal bovine serum in TNT

Blocking buffer can be stored at 4°C and reused several times. Sodium azide should be added to a final concentration of 0.05% to inhibit the growth of microorganisms.

Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

Other possible blocking agents include 1% gelatin, 3% bovine serum albumin, or 5% nonfat dried milk in TNT. Opinion about which of these agents is best varies from laboratory to laboratory. We recommend carrying out preliminary experiments to determine which of them works best in your particular circumstances.

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12. Using blunt-ended forceps, transfer the filters to fresh glass trays or petri dishes containing the primary antibody diluted in blocking buffer (7.5 ml for each 82-mm filter; 15 ml for each 138-mm filter). Use the highest dilution of antibody that gives acceptable background yet still allows detection of 50–100 pg of denatured antigen. When all of the filters have been submerged, agitate the solutions gently on a rotary platform for 2–4 hours at room temperature.

    The antibody solution can be stored at 4°C and reused several times. Sodium azide (see Caution above) should be added to a final concentration of 0.05% to inhibit the growth of microorganisms.

13. Wash the filters for 10 minutes in each of the buffers below in the order given. Transfer the filters individually from one buffer to the next. Use 7.5 ml of each buffer for each 82-mm filter and 15 ml for each 138-mm filter.

    TNT + 0.1% bovine serum albumin
    TNT + 0.1% bovine serum albumin + 0.1% Nonidet P-40
    TNT + 0.1% bovine serum albumin

    These buffers do not contain sodium azide.

14. Detect the antigen-antibody complexes with the radiochemical or chromogenic reagent of your choice:

    - Use approximately 1 μCi of ^{125}I-labeled protein A or immunoglobulin per filter. Radiolabeled protein A is available from commercial sources (sp. act. 30 mCi/mg). Radioiodinated second antibody can be prepared as described on page 12.29. Dilute radiolabeled ligands in blocking buffer (7.5 ml for each 82-mm filter; 15 ml for each 138-mm filter). Incubate the filters for 1 hour at room temperature, and then wash them several times in TNT before establishing autoradiographs as described in Appendix E.

    - Antibodies coupled to HRP or AP that react with species-specific determinants on primary antibodies are available from commercial sources and should be used at the recommended dilution in accordance with the manufacturer's instructions. Typically, 5 μl of conjugated antiserum is used for each 82-mm filter in 7.5 ml of blocking buffer (without sodium azide). Gently agitate the filters for 1.5–2 hours in the antibody solution at room temperature, and then wash them as described in step 13.

      Antigen-antibody–antibody-HRP complexes are located using a chromogenic substrate such as 4-chloro-1-naphthol. To prepare developing solution, dissolve 60 mg of this substrate in 20 ml of ice-cold methanol. Just before use, mix the solution with 100 ml of 10 mM Tris · Cl (pH 7.5), 150 mM NaCl containing 60 μl of 30% H_2O_2. After the last wash following exposure to the second antibody, blot the filters on paper towels, wash them briefly in 10 mM Tris · Cl (pH 7.5), 150 mM NaCl and incubate them for 15–20 minutes at room temperature in the 4-chloro-1-naphthol developing solution described above (10 ml for each

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82-mm filter; 25 ml for each 138-mm filter). Then wash the filters in two changes of distilled water. An intense purple color will develop at the site of antigen-antibody complexes.

Antigen-antibody–antibody-AP complexes are located using the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in combination with nitro blue tetrazolium (NBT), which detects the precipitated indoxyl group. Stock solutions of these compounds are stable when stored in the dark (BCIP, 50 mg/ml in 100% dimethylformamide; NBT, 50 mg/ml in 70% dimethylformamide). Just before use, prepare the BCIP/NBT developing solution as follows: Add 33 μl of the NBT solution to 5 ml of AP buffer (100 mM Tris · Cl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$), mix, and then add 16.5 μl of the stock solution of BCIP. Mix again. Protect the solution from strong light and use it within an hour. After the last wash following exposure to the second antibody, blot the filters on paper towels and incubate them in the BCIP/NBT developing solution (7.5 ml for each 82-mm filter; 15 ml for each 138-mm filter) at room temperature for several hours. Then rinse the filters briefly in two changes of distilled water. An intense purple color will develop at the site of antigen-antibody complexes.

Biotinylated species-specific antibodies and avidin-conjugated HRP are available from commercial sources. They should be diluted according to individual manufacturer's instructions and used for immunological screening as described above for HRP-conjugated antibodies.

15. Identify the locations of positive plaques or, if made, compare the duplicate filters, searching for coincident signals.

a. Lay a sheet of Saran Wrap over the filters.

b. On the surface of the Saran Wrap, mark the locations of the holes in the filters and the locations of antigen-positive clones with different colored waterproof markers. Label the Saran Wrap so that you can identify the plates from which the filters were derived.

c. Place the sheet of Saran Wrap on a light box, and align the plates containing the original bacteriophage λ plaques on top of it.

d. Identify the area containing the positive plaque, and remove a plug of agar from this area using the large end of a pasteur pipette. Transfer the plug to 1 ml of SM containing 2 drops of chloroform.

e. Keep the sheet of Saran Wrap, which provides a permanent record of the locations of the positive plaques. The purple spots on the original filters fade quite rapidly.

16. Allow the bacteriophage particles to elute from the agar plug for several hours at 4°C. Determine the titer of the bacteriophages in the eluate, and then replate them so as to obtain approximately 3000 plaques per 90-mm plate. Rescreen the plaques as described above, and repeat the process of screening and plating until a homogeneous population of immunopositive recombinant bacteriophages is obtained.

SCREENING BACTERIAL COLONIES

Preparation of Colonies for Screening

METHOD 1

This procedure (Hanahan and Meselson 1980) is used when you know in advance that you will need to screen large numbers of colonies. In this case, bacteria are plated directly from a transformation mixture onto detergent-free nitrocellulose filters (Millipore HATF or equivalent); replica filters are prepared by filter-to-filter contact.

1. Number the nitrocellulose filters with a soft-lead pencil or ballpoint pen, wet them with water, and sandwich them between dry Whatman 3MM papers. Wrap the stack of filters in aluminum foil, and sterilize them by autoclaving at 10 lb/sq. in. on liquid cycle.

2. Using sterile blunt-ended forceps (e.g., Millipore forceps), lay a sterile filter, numbered side down, on a day-old LB (or SOB) plate containing the appropriate antibiotic. Remove the filter from the plate, invert it, and replace it, numbered side up.

3. Apply the bacteria in a small volume of liquid (< 0.5 ml containing up to 20,000 bacteria for a 138-mm filter; < 0.2 ml containing up to 10,000 bacteria for an 82-mm filter). Spread the liquid over the surface of the filter with a sterile bent glass rod. Leave a border 2–3 mm wide at the edge of the filter free of bacteria. Let the plates stand at room temperature until all of the liquid has been absorbed.

4. Invert the plates and incubate them at 37°C (or 30°C) until very small (0.1-mm diameter) colonies appear (8–10 hours).

   Colonies containing expression vectors carrying the *lac* promoter should be grown at 37°C. Colonies containing expression vectors carrying the bacteriophage λ p_R promoter should be grown at 30°C to prevent the expression of fusion proteins.

5. Prepare in advance a stack of 3MM papers (one for each filter plus a few spares) sterilized by autoclaving (see step 1).

6. Wet a numbered, sterile nitrocellulose filter (Millipore HAWP or equivalent) by touching it, numbered side up, to the surface of a fresh agar plate containing the appropriate antibiotic. Leave the filter in contact with the surface of the agar. The numbers on these replica filters should correspond to those on the master filters.

7. Using sterile blunt-ended forceps, gently remove the master filter from the first plate and place it, colony side up, on the stack of 3MM papers.

8. Carefully place the second, correspondingly numbered, wetted filter, numbered side down, on top of the master, being careful not to move the filters once contact has been made. Press the filters together using a sterile velvet replica-plating tool (see Figure 12.1).

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9. Using an 18-gauge needle, make a characteristic set of registration holes in the filters while they are sandwiched together. Gently peel the filters apart, and place them, colony side up, on fresh agar plates containing the appropriate antibiotic.

   If required, several replicas can be made from a single master filter. However, if the master filter is to be used to make more than two replicas, it should be reincubated for a few hours to allow the colonies to regenerate. Generally, it is best to make only two replicas from a single master to avoid problems caused by smearing of the colonies.

10. To induce the expression of a gene cloned into a plasmid carrying the *lac* promoter, incubate the plates (masters and replicas) at 37°C until colonies 1–2 mm in diameter have appeared. Colonies on the master plate reach the desired size more rapidly. (Store the master plate as described in step 12.) At this stage, transfer the replica filter, numbered side up, to another plate that contains isopropylthio-β-D-galactoside (IPTG). Incubate a further 2–4 hours.

    To induce synthesis in expression vectors that carry the bacteriophage λ p_R promoter (e.g., the pEX vectors; see Chapter 17), transfer the filter to a prewarmed plate and incubate for 2–4 hours at 42°C.

11. Remove the filter and process it for immunological screening as described on page 12.24.

12. Wrap the master plate in Saran Wrap, and store it at 4°C in an inverted position until the results of immunological screening are available.

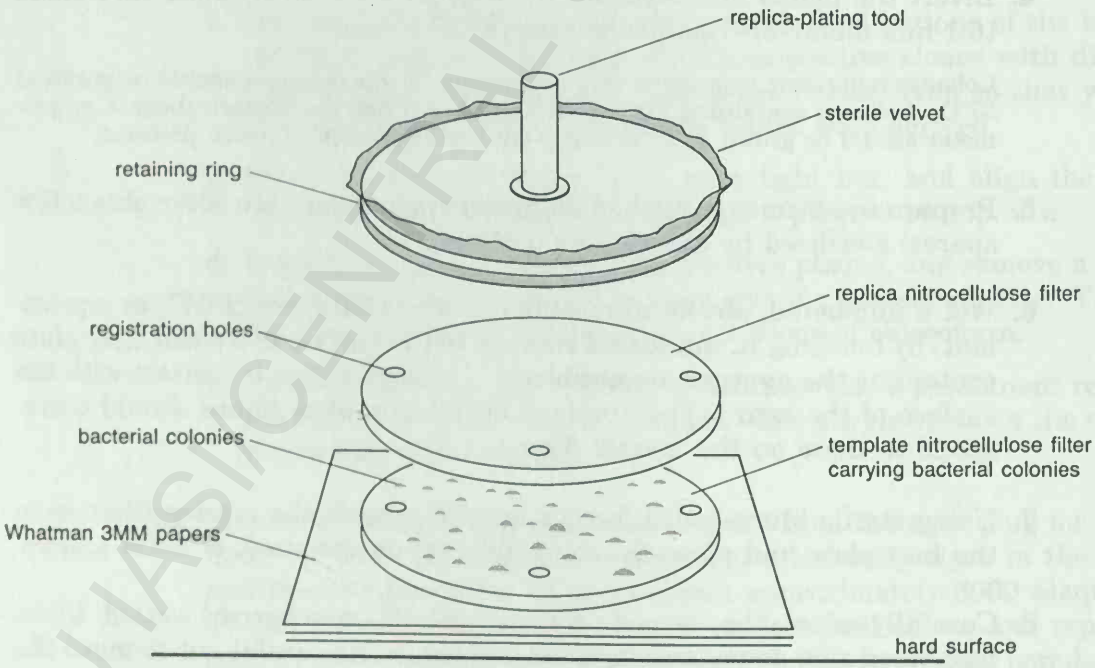

FIGURE 12.1
Use of a replica-plating tool to replicate bacterial colonies growing on nitrocellulose filters.

This method is used to transfer many bacterial colonies simultaneously from the surface of an agar plate to a nitrocellulose filter. The method works with bacterial colonies of any size, but small colonies (0.1–0.2 mm) give the best results: They produce sharper signals and smear less than larger colonies. As many as 2×10^4 colonies per 150-mm plate can be screened by this technique.

Note: Colonies containing expression vectors carrying the *lac* promoter should be grown at 37°C. Colonies containing expression vectors carrying the bacteriophage λ p_R promoter should be grown at 30°C to prevent the expression of fusion proteins.

1. After the bacterial colonies have grown to a diameter of 0.1–0.2 mm, remove the plate from the incubator and store it for 1–2 hours at 4°C in an inverted position.

2. Label a dry, sterile nitrocellulose filter (Millipore HAWP or equivalent) with a soft-lead pencil or ballpoint pen and place it, numbered side down, on the surface of the agar medium, in contact with the bacterial colonies, until it is completely wet. Mark the filter in at least three asymmetric locations by stabbing through it and into the agar underneath with an 18-gauge needle attached to a syringe containing waterproof black ink.

3. To induce the expression of a gene cloned into a plasmid carrying the *lac* promoter, transfer the filter, numbered side up, to a fresh agar plate containing isopropylthio-β-D-galactoside (IPTG). Incubate the plate for 2–4 hours at 37°C.

   To induce synthesis in expression vectors that carry the bacteriophage λ p_R promoter (e.g., the pEX vectors; see Chapter 17), transfer the filter to a prewarmed plate and incubate for 2–4 hours at 42°C.

4. Remove the filter, and process it for immunological screening as described on page 12.24.

5. Incubate the master plate for 6 hours at 37°C (or 30°C) to allow the colonies to regenerate. Wrap the plate in Saran Wrap and store it at 4°C in an inverted position until the results of immunological screening are available.

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Processing Filters for Immunological Screening of Colonies

Caution: Step 1 should be carried out in a chemical hood.

1. Using blunt-ended forceps (e.g., Millipore forceps), remove the nitrocellulose filters from the plates and place them on damp paper towels. Cover the filters with a plastic hood of the kind used to cover food in delicatessens. Place in the plastic hood an open glass petri dish containing chloroform. Expose the bacterial colonies on the filters to chloroform vapor for 15 minutes.

2. Transfer small groups of filters to petri dishes containing lysis buffer (6 ml per 82-mm filter; 12 ml per 138-mm filter). When all of the filters have been submerged, stack the petri dishes on a rotary platform and agitate the lysis buffer by gentle rotation of the platform. Lysis of the bacterial colonies takes 12–16 hours at room temperature.

Lysis buffer

100 mM Tris · Cl (pH 7.8)
150 mM NaCl
5 mM $MgCl_2$
1.5% bovine serum albumin
1 μg/ml pancreatic DNAase I
40 μg/ml lysozyme

3. Transfer the filters to petri dishes or glass trays containing TNT. Incubate for 30 minutes at room temperature.

TNT

10 mM Tris · Cl (pH 8.0)
150 mM NaCl
0.05% Tween 20

4. Repeat step 3 using fresh TNT.

5. Transfer the filters, one by one, to a glass tray containing TNT. Use Kimwipes to wipe off the residue of the colonies from the surfaces of the filters.

Important: Do not allow the filters to dry during any of the subsequent steps. Antibodies bound nonspecifically and reversibly to wet filters become permanently attached if the filters dry out. It is also essential that the filters do not stick to one another when they are immersed in the various buffers and antibody solutions. This problem can be minimized by dividing the filters into small batches (five filters per batch) and using a separate large petri dish for each batch. The petri dishes can be stacked on top of one another on a slowly rotating platform shaker.

6. Continue to process the filters for immunological screening as described on page 12.18, beginning at step 10.

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1. On ten agar plates, plate out nonrecombinant bacteriophage λ (if possible, using the vector in which the expression library was constructed) so as to obtain semiconfluent lysis of the bacterial lawn.

2. Prepare imprints of the lysed lawns on nitrocellulose filters as described in steps 4–10, pages 12.16–12.18, but omitting IPTG.

3. Incubate the filters for 6 hours with the preparation of antibody (diluted 1:10 in TNT containing 1% gelatin, 3% bovine serum albumin, or 5% nonfat dried milk) that is to be used for screening. The absorbed antibody may be stored at 4°C in the presence of 0.05% sodium azide until used for immunological screening.

   Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

Notes

i. This method of absorption only works for preparations of antiserum that contain relatively low titers of anti-*E. coli* antibodies. If the absorbed antiserum continues to give an unacceptably high level of reaction with host components, it should be further purified by chromatography on a column that contains immobilized *E. coli* components (de Wet et al. 1984) (see page 12.27).

ii. This method can easily be adapted to remove anti-*E. coli* antibodies by pseudoscreening of bacterial colonies. On nitrocellulose filters, establish bacterial colonies that carry the expression vector without an insert of DNA. Then follow the screening procedure described on page 12.24.

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PREPARATION OF E. coli LYSATES FOR ABSORPTION OF ANTI-E. coli ANTIBODIES

1. Grow 100-ml cultures of *E. coli* strain Y1090*hsdR* to saturation in LB medium.

2. Harvest the cells by centrifugation at 5000*g* for 10 minutes at 4°C.

3. Resuspend the cells in 3 ml of 50 mM Tris · Cl (pH 8.0), 10 mM EDTA (pH 8.0). Freeze and thaw the suspension several times, and then sonicate it at full power for six periods of 20 seconds each at 0°C.

4. Centrifuge the extract at 12,000*g* for 10 minutes at 4°C. Transfer the supernatant to a fresh tube. Store the lysate at −20°C.

5. Use the lysate as follows: Dilute the antiserum 1:10 with TNT containing 1% gelatin, 3% bovine serum albumin, or 5% nonfat dried milk. Add 0.5 ml of lysate for every milliliter of antiserum used. Incubate the mixture for 4 hours at room temperature. The absorbed antibody may be stored at 4°C in the presence of 0.05% sodium azide until used for immunological screening.

   Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

Note

This method of absorption only works for preparations of antiserum that contain moderate titers of anti-*E. coli* antibodies. If the absorbed antiserum continues to give an unacceptably high level of reaction with host components, it should be further purified by chromatography on a column that contains immobilized *E. coli* components (de Wet et al. 1984) (see page 12.27).

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REMOVAL OF ANTI-E. coli ANTIBODIES BY AFFINITY CHROMATOGRAPHY

This method is adapted from de Wet et al. (1984).

1. Grow 1-liter cultures of the appropriate strain of *E. coli* (e.g., Y1090*hsdR*, XL1-Blue, or DH1) to stationary phase.

2. Recover the bacteria by centrifugation at 5000 rpm for 20 minutes at 4°C in a Sorvall HS-4 rotor (or its equivalent).

3. Pour off the medium, and stand the centrifuge tubes in an inverted position to allow the last traces of medium to drain away.

4. Resuspend the pellet from 1 liter of the original culture in 100 ml of 0.1 M sodium borate (pH 8.0), 1 M NaCl.

5. Add 200 mg of lysozyme, and incubate the cell suspension for 20 minutes at room temperature.

6. Add 1 mg of pancreatic DNAase I and 200 μl of Triton X-100.

7. Incubate the cell supension for 1 hour at 4°C, or until the turbidity clears and the viscosity decreases.

8. Centrifuge the bacterial lysate at 8000*g* for 20 minutes at 4°C. Carefully decant the supernatant into a fresh flask.

9. Adjust the pH of the supernatant to 9.0 with 1 N NaOH.

10. Chill the extract to 0°C, and then bind the bacterial proteins to cyanogen-bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

11. Before use, equilibrate the Sepharose 4B containing *E. coli* proteins in Tris-buffered saline (TBS; see Appendix B) containing 0.2% sodium azide.

    Caution: Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

12. Use 1 ml of settled volume of Sepharose 4B coupled to *E. coli* antigen for each milligram of IgG to be purified by affinity chromatography. Mix the IgG and the coupled Sepharose 4B and incubate for 12–18 hours at room temperature on a tumbling device.

    IgG should be purified from antiserum as described by Goudswaard et al. (1978).

13. Load the slurry into a 5-ml plastic syringe plugged with glass wool. Recover the antibody by washing the column with TBS. Collect fractions

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(0.2 column volume each) until the OD_{280} drops to zero. Pool the fractions containing antibody, and store the affinity-purified antibody at −20°C until it is used for immunological screening.

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RADIOIODINATION OF IMMUNOGLOBULIN G

Caution: The following procedure should be carried out behind appropriate shielding in a chemical hood that is correctly vented.

1. Prepare IgG from antiserum as described by Goudswaard et al. (1978). Dilute the IgG with 50 mM sodium phosphate (pH 7.5) to obtain a final concentration of 1 mg/ml.

2. To 50 μl of the diluted IgG, add 4 μl of ^{125}I (sodium ^{125}iodide, 10 mCi/ml).

3. Add 15 μl of chloramine T (2 mg/ml in 50 mM sodium phosphate [pH 7.5]).

4. Incubate for 90 seconds at room temperature.

5. Add 50 μl of sodium metabisulfite (5 mg/ml in 50 mM sodium phosphate [pH 7.5]).

6. Apply the solution to a 5-ml column of Sephadex G-25 (equilibrated in 50 mM sodium phosphate [pH 7.5]) (see Appendix E).

7. Collect 0.5-ml fractions until all of the radioactivity has eluted (~3 ml).

8. Pool the radioactive material. Usually, more than 95% of the radiolabel is incorporated and the specific activity of the radiolabeled antibody exceeds 10^8 cpm/mg. Do not store radiolabeled antibody for prolonged periods before use.

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Screening cDNA Libraries Constructed in Bacteriophage λ Expression Vectors with Synthetic Oligonucleotides

cDNA libraries constructed in bacteriophage λ expression vectors can be screened with synthetic oligonucleotides to identify clones corresponding to specific DNA-binding proteins (Singh et al. 1988; Vinson et al. 1988). The methods are very similar to those used for immunological screening of expression libraries, except that the nitrocellulose filters carrying immobilized proteins are screened with ^{32}P-labeled double-stranded DNA rather than with antibodies. Initially, screening was carried out with radiolabeled double-stranded DNA carrying a single binding site (Singh et al. 1988). However, under these circumstances, the signal from a positive clone was often weak and was easily washed from the filter. The equilibrium association binding constants of sequence-specific DNA-binding proteins range from 10^8–10^{12} M^{-1}. Assuming that a protein has an equilibrium association binding constant of 10^{10} M^{-1} and an association rate constant of 10^7 $M^{-1} \cdot s^{-1}$, the dissociation rate constant of the resulting DNA–protein complex will be 10^{-3} s^{-1} and its half-life will be approximately 10 minutes (Singh et al. 1988). Thus, only one eighth of the complexes will survive a 30-minute wash. This calculation therefore indicates that screening of expression libraries with double-stranded oligonucleotide probes is unlikely to be successful if the equilibrium association binding constant of the DNA-binding protein is less than 10^9 M^{-1}. This problem can be alleviated by using a concatenated probe that contains multiple binding sites (Clerc et al. 1988; Staudt et al. 1988; Vinson et al. 1988). In side-by-side comparisons, a much stronger signal was generated with probes containing multiple binding sites, presumably because the probe is tethered at more than one site to the DNA-binding protein produced in the bacteriophage plaque. Because of this observation, double-stranded oligonucleotides are now routinely ligated to form concatemers prior to screening. Using such concatenated probes that have been radiolabeled to a specific activity in excess of 10^8 cpm/μg, it is possible to detect as little as 1 pg of a β-galactosidase fusion protein that contains a DNA-binding site.

Plaques generated by recombinant λgt11 bacteriophages typically contain 50–100 pg of fusion protein. However, only a small proportion of the protein produced by the bacteriophage-infected cells would normally be folded into a conformation suitable for binding to its DNA ligand. This situation can sometimes be improved by exposing the fusion protein briefly to denaturing agents (Vinson et al. 1988). Nitrocellulose filters carrying the fusion proteins synthesized by recombinant bacteriophages are soaked in 6 M guanidine HCl. During this process, aggregates of fusion proteins are solubilized and partially denatured. The denaturant is then gradually removed, allowing an increased proportion of the fusion protein to refold into a conformationally active form. However, it is important to note that exposure to denaturant is not always beneficial (P. Sharp, pers. comm.), apparently because some proteins are not readily renatured after treatment with guanidine HCl. The

safest course of action, therefore, is to screen two sets of filters in parallel, only one of which has been exposed to guanidine HCl. In some cases, it is possible to determine in preliminary experiments whether the protein of interest will survive denaturation and renaturation. For example, nuclear extracts containing the protein of interest can be exposed to a cycle of denaturation and renaturation and then assayed in methylation protection experiments (Siebenlist et al. 1980; see also Kadonaga et al. 1987), by DNAase I footprinting (Galas and Schmitz 1978), or in gel electrophoresis DNA-binding assays (gel retardation assays) (Fried and Crothers 1981; Garner and Revzin 1981; see also Chodosh 1988).

Potential positive plaques that react strongly with double-stranded oligonucleotide probes are picked, replated at appropriate dilutions, and retested for their ability to react with both specific and nonspecific concatenated oligonucleotide probes. If possible, the nonspecific probes used in this second round of screening should be generated from oligonucleotides that contain base substitutions known to disrupt protein–DNA interaction either in vivo or in vitro. Clones scoring as positive in this second round of screening are used to generate lysogenic strains of bacteria that are induced to synthesize large amounts of protein for use in the types of biochemical assays outlined above. Ultimately, the functionality of the protein must be demonstrated by in vitro transcription studies or in cotransfection experiments in which a plasmid carrying the full-length cloned cDNA is introduced into mammalian cells together with a reporter plasmid containing multiple copies of the recognition sequence upstream of a promoter. The ability of the protein encoded by the cDNA to activate or stimulate transcription from the promoter can then be assayed by analysis of RNA extracted from the transfected cells.

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Preparing Radiolabeled Concatenated Probes

Radiolabeled probes are prepared by phosphorylating the 5′ termini of two complementary synthetic oligonucleotides, annealing the phosphorylated DNAs, and ligating the annealed DNAs to form concatemers. The double-stranded concatemers are then radiolabeled by nick translation. The following method was provided by S. McKnight.

1. Set up two separate phosphorylation reactions, each containing one of the synthetic oligonucleotides:

synthetic oligonucleotide (in 10 μl of TE [pH 7.6])	2.0 μg
10 × kinase/ligase buffer	5.0 μl
100 mM dithiothreitol	2.5 μl
10 mM ATP	5.0 μl
H_2O	23.0 μl
bacteriophage T4 polynucleotide kinase (8 units/μl)	2.0 μl

Incubate the reactions for 1 hour at 37°C.

10 × Kinase/ligase buffer

500 mM Tris · Cl (pH 7.6)
100 mM $MgCl_2$

2. Mix the two phosphorylation reactions together. Anneal the oligonucleotides by incubating the mixture as follows:

2 minutes at 85°C
15 minutes at 65°C
15 minutes at 37°C
15 minutes at room temperature
15 minutes on ice

3. Add 4 μl of bacteriophage T4 DNA ligase (1 Weiss unit/μl) and 1 μl of 50 mM ATP. Incubate the mixture for 12 hours at 16°C.

At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units (as defined by New England Biolabs). 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units.

4. Add 0.5 M EDTA (pH 8.0) to a final concentration of 5 mM and 20% SDS to a final concentration of 0.5%. Extract the mixture once with phenol: chloroform, and transfer the aqueous phase to a fresh tube.

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5. Add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M, and then add 3 volumes of ethanol at 0°C. Store the mixture on ice for 30 minutes, and then recover the DNA by centrifugation at 12,000*g* for 15 minutes at 0°C. Wash the precipitate with 500 μl of 70% ethanol at 0°C, and recentrifuge for 2 minutes. Remove the supernatant by aspiration, and allow the pellet of DNA to dry at room temperature.

6. Redissolve the DNA in 24 μl of TE (pH 7.6).

7. Radiolabel 500 ng of the annealed, ligated DNA by nick translation (see Chapter 10, pages 10.6–10.12). The specific activity of the final probe should be 2×10^8–5×10^8 cpm/μg.

8. Analyze the size of the radiolabeled DNA by nondenaturing polyacrylamide gel electrophoresis (see Chapter 6) and autoradiography (see Appendix E). If all has gone well, the radiolabeled concatenated DNA should form a ladder.

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Preparation of Filters for Screening with Radiolabeled Concatenated Probes

Plates and nitrocellulose filters are prepared in exactly the same way as described on pages 12.16–12.17, steps 1–8. The filters are then processed and screened as described below. The following method was provided by S. McKnight.

1. Remove the nitrocellulose filters from the lawn of plaques with blunt-ended forceps (e.g., Millipore forceps) and place them on Whatman 3MM paper with the side exposed to the plaques facing upward. Allow the filters to dry for 15 minutes at room temperature.

2. Lay a second (numbered) filter impregnated with IPTG on each lawn (see page 12.16). With an 18-gauge needle, make holes in each filter in the same locations as those used to key the first filter to the lawn. Incubate the plates for 2 additional hours at 37°C, and then remove the filters and allow them to dry at room temperature as described in step 1.
   Important: All subsequent steps should be carried out at 4°C.

3. Divide the filters into two sets. One set is probed directly without denaturation with guanidine HCl (i.e., omitting steps 4–6), whereas the other set is processed as described in steps 4–6.

4. Place the filters in a 12-in. × 8-in. baking dish containing 6 M guanidine HCl in 1 × binding buffer (denaturation solution: 15 ml for each 82-mm filter; 25 ml for each 138-mm filter) at 4°C. Agitate the filters gently on a platform shaker for 5 minutes at 4°C. Decant the denaturation solution, and replace it with fresh solution. Agitate the filters for an additional 5 minutes at 4°C.

   Denaturation solution is made by diluting 10 × binding buffer with five volumes of distilled water. This solution is then added to the appropriate amount of solid guanidine HCl. When the guanidine HCl has completely dissolved, adjust the concentration of the binding buffer to 1 × with distilled water and add dithiothreitol to a final concentration of 1 mM.

   10 × Binding buffer

   250 mM HEPES (pH 7.9)
   30 mM $MgCl_2$
   40 mM KCl

5. Decant the second batch of denaturation solution into a graduated cylinder. Dilute the solution with an equal volume of 1 × binding buffer containing 1 mM dithiothreitol. Pour this solution into a clean glass dish, and transfer the filters to the solution one at a time, making sure that each filter becomes completely exposed to the more dilute denaturation solution.

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6. Repeat the process described in step 5 four more times, diluting the denaturation solution by a factor of two each time. The concentrations of guanidine HCl in the solutions are therefore 3 M (step 5), 1.5 M, 0.75 M, 0.375 M, and 0.187 M. Finally, wash the filters twice in 1 × binding buffer containing 1 mM dithiothreitol.

7. Place both sets of filters in 1 × binding buffer containing 5% nonfat dried milk. Agitate the filters gently for 30 minutes at 4°C.

8. Rinse the filters in 1 × binding buffer containing 0.25% nonfat dried milk.

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Probing Immobilized Proteins with Radiolabeled DNA

The following method was provided by S. McKnight.

1. In a crystallizing dish, add the ^{32}P-labeled concatenated DNA probe (see pages 12.32–12.33) to 1 × binding buffer (see page 12.34) containing 1 mM dithiothreitol, 0.25% nonfat dried milk, and 10 μg/ml sonicated, denatured salmon sperm or calf thymus DNA. Use 10 ml of buffer for each 82-mm filter or 25 ml for each 138-mm filter. Optimal results are usually obtained when the final concentration of probe (sp. act. 2×10^8–5×10^8 cpm/μg) is approximately 25 ng/ml. However, concentrations as low as 2.5 ng/ml can be used if the amount of probe is limiting.

   In the original description of this method (Singh et al. 1988), poly(dI · dC)-poly(dI · dC) was used as the nonspecific competitor. However, lower backgrounds are generally achieved when sonicated, denatured salmon sperm or calf thymus DNA is used. A method for the preparation of this DNA is given in Appendix B.

2. Transfer the filters prepared in steps 1–8, pages 12.34–12.35, to the radiolabeled-probe solution in the crystallizing dish. Incubate with gentle agitation on a rotating platform for 2–12 hours at 4°C.

   Although binding buffer is suitable for most DNA–protein interactions, in some cases it may be necessary to adjust the concentrations of salt and magnesium ions to conform to the optimal conditions for binding established in in vitro tests with nuclear extracts.

3. Wash the filters for 5 minutes at 4°C in a large volume (25 ml for each 82-mm filter; 60 ml for each 138-mm filter) of 1 × binding buffer containing 1 mM dithiothreitol and 0.25% nonfat dried milk.

4. Repeat step 3 twice more.

5. Decant the final wash buffer. Arrange the filters on a sheet of Saran Wrap. Cover the filters with another sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filters. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

   Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot (> 2000 cps on a hand-held minimonitor), hot (> 500 cps on a hand-held minimonitor), and cool (> 50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

6. Establish an autoradiograph as described in Appendix E.

7. Pick positive plaques, and rescreen them with specific and nonspecific probes as discussed on page 12.31.

Notes

i. It is highly desirable to use an unamplified expression library for screening by this method. If the protein of interest is toxic to bacteria or suppresses growth of the bacteriophage, the clone will be eliminated or underrepresented in amplified libraries.

ii. Filters may be reprobed by repeating the denaturation/renaturation cycle (pages 12.34–12.35), which effectively removes the probe from the filters.

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Preparation of Lysates Containing Fusion Proteins Encoded by Bacteriophage λgt11 Lysogens

The isolation of recombinant bacteriophages encoding fusion proteins that bind to double-stranded oligonucleotide probes is obviously an important step toward the cloning of cDNAs that code for transcription factors. However, further work is required to confirm that the proteins specified by these bacteriophages bind specifically to regulatory regions of the appropriate genes. The first step in this process is to construct a bacteriophage lysogen from the bacteriophage λ expressing the DNA-binding protein. The fusion protein of interest is then isolated from this lysogen and tested for the presence of specific DNA-binding proteins. The construction of a lysogen and the isolation of fusion proteins are described below (modified from Snyder et al. [1987] and Singh et al. [1989]). Various procedures for testing the functionality of the DNA-binding protein are listed in step 21.

1. Make a plate stock of each of the recombinant bacteriophages of interest using the methods described in Chapter 2. The titer of the stocks, measured on *E. coli* strain Y1090*hsdR*, should be $>10^{10}$ pfu/ml.

2. Grow a 2-ml culture of *E. coli* strain Y1089 to saturation in LB medium containing 0.2% maltose, 50 μg/ml ampicillin, and 10 mM $MgCl_2$.

3. Dilute 50 μl of the saturated culture with 2 ml of LB medium containing 10 mM $MgCl_2$. Transfer four 100-μl aliquots of the diluted culture to fresh culture tubes.

4. To three of the tubes, add 1×10^7, 5×10^7, or 2×10^8 pfu of the bacteriophage stock. The fourth tube should receive no bacteriophage. Incubate the four tubes for 20 minutes at room temperature.

5. Dilute 10 μl of each of the four cultures with 10 ml of LB medium. Immediately plate aliquots (100 μl) of each of the four diluted cultures onto LB plates containing 50 μg/ml ampicillin. Incubate the plates for 18–24 hours at 30°C.

   No colonies should appear on the plates inoculated with the uninfected culture of *E. coli* Y1089. Infected cultures should give rise to 50–500 colonies, depending on the density of the original saturated culture and on the exact multiplicity of bacteriophage infection.

6. Using sterile toothpicks, transfer individual colonies onto two LB plates that contain 50 μg/ml ampicillin. Incubate one plate at 30°C and the other at 42°C for 12–16 hours. Clones that give rise to colonies at 30°C but not at 42°C are lysogenic for recombinant bacteriophage λgt11. Usually 10–70% of the colonies tested are lysogens.

7. Inoculate 2 ml of LB medium containing 50 μg/ml ampicillin with individual bacteriophage λgt11 lysogens. Grow the cultures for 12–16 hours at 30°C with vigorous agitation (300 cycles/minute in a rotary shaker).

8. Add 50 μl of each culture to 4 ml of prewarmed (30°C) LB medium containing 50 μg/ml ampicillin. Continue incubation at 30°C with vigorous agitation.

9. Grow the cultures until the $OD_{600} = 0.45$ (~3 hours of incubation).

   It is essential that the OD_{600} of the cultures should not exceed 0.5 before the lysogens are induced.

10. Transfer the cultures to a water bath equilibrated to 44°C. Incubate the cultures for 15 minutes at 44°C.

    Maximal efficiency of induction is obtained by rapidly raising the temperature from 30°C to 44°C. It is therefore preferable to transfer the cultures to a water bath set at 44°C than to a hot-air incubator. Shake the cultures vigorously from time to time during incubation at 44°C.

11. Add isopropylthio-β-D-galactoside (IPTG) to each culture to a final concentration of 10 mM, and then incubate the cultures for 1 hour at 37°C with vigorous agitation.

12. Transfer each of the induced lysogenic cultures to two microfuge tubes. Immediately centrifuge the tubes at 12,000*g* for 30 seconds at 4°C in a microfuge.

13. Remove the medium by aspiration, and then rapidly resuspend the bacterial pellets by vortexing in 100 μl of lysogen extraction buffer.

Lysogen extraction buffer

50 mM Tris · Cl (pH 7.5)
1 mM EDTA (pH 8.0)
5 mM dithiothreitol
50 μg/ml phenylmethylsulfonyl fluoride (PMSF)

PMSF should be added to the lysogen extraction buffer just before it is used.

Caution: PMSF is extremely destructive to the mucous membranes of the respiratory tract, the eyes, and skin. It may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water. Discard contaminated clothing.

PMSF is inactivated in aqueous solutions. The rate of inactivation increases with pH and is faster at 25°C than at 4°C. The half-life of a 20 μM aqueous solution of PMSF is about 35 minutes at pH 8.0 (James 1978). This means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH > 8.6) and stored for several hours at room temperature. PMSF is usually stored as a 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml in isopropanol) at −20°C.

14. Close the caps of the tubes, and place the tubes in liquid nitrogen.

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15. After 2 minutes, recover the tubes from the liquid nitrogen. Hold the tubes in your hand until the lysates just thaw, and then immediately add to each tube 20 μl of a freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tris·Cl [pH 8.0]). Store the tubes for 15 minutes in an ice bath.

16. Add 250 μl of 5 M NaCl to each tube. Mix the contents by flicking the side of each tube with a finger. Incubate the tubes for 30 minutes at 4°C on a rotating wheel.

17. Centrifuge the tubes at 12,000*g* for 30 minutes at 0°C in a microfuge.

18. During the centrifugation step, float a Millipore filter (142 mm, type VS, 0.025-micron pore size) on the surface of a petri dish (150 mm) filled with lysogen extraction buffer at 4°C.

    This step is best carried out in a quiet corner of a coldroom. Take care that no lysogen extraction buffer spills over onto the upper surface of the filter.

19. Transfer the supernatants from the centrifuge tubes to the upper surface of the filter. Up to 20 different samples can be applied to the same filter.

20. After 1–2 hours at 4°C, transfer the dialyzed samples to fresh microfuge tubes, which can be stored at −70°C until needed.

21. Test the lysates for the presence of DNA-binding proteins, for example, in methylation protection experiments (Siebenlist et al. 1980; see also Kadonaga et al. 1987), by DNAase I footprinting (Galas and Schmitz 1978), or in gel electrophoresis DNA-binding assays (gel retardation assays) (Fried and Crothers 1981; Garner and Revzin 1981; see also Chodosh 1988).

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13
DNA Sequencing

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In the mid-1970s, when molecular cloning techniques in general were rapidly improving, simple methods were also developed to determine the nucleotide sequence of DNA. These advances laid the foundation for the detailed analysis of the structure and function of large numbers of genes. The first attempts to sequence DNA mirrored techniques developed in the 1960s to sequence RNA (see Sanger et al. 1965; Brownlee et al. 1968; Brownlee 1972). These involved (1) specific cleavage of the DNA into smaller fragments by enzymatic digestion (endonuclease IV [Robertson et al. 1973; Ziff et al. 1973]) or chemical digestion (pyrimidine tract analysis [Robertson et al. 1973; Ziff et al. 1973]), (2) nearest neighbor analysis (Wu and Taylor 1971), and (3) the wandering spot method (Sanger et al. 1973; Tu and Wu 1980). Indeed, in some studies the DNA was transcribed into RNA with *Escherichia coli* RNA polymerase and then sequenced as RNA (Gilbert and Maxam 1973). It is a testimony to the success of DNA sequencing that today most protein sequences are deduced from the nucleotide sequences of genes or cDNAs.

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Sequencing Techniques and Strategies

The two rapid sequencing techniques in current use are the enzymatic method of Sanger et al. (1977) and the chemical degradation method of Maxam and Gilbert (1977). Although very different in principle, these two methods both generate separate populations of radiolabeled oligonucleotides that begin from a fixed point and terminate randomly at a fixed residue or combination of residues. Because every base in the DNA has an equal chance of being the variable terminus, each population consists of a mixture of oligonucleotides whose lengths are determined by the location of a particular base along the length of the original DNA. These populations of oligonucleotides are then resolved by electrophoresis under conditions that can discriminate between individual DNAs that differ in length by as little as one nucleotide. When the populations are loaded into adjacent lanes of a sequencing gel, the order of nucleotides along the DNA can be read directly from an autoradiographic image of the gel (see, e.g., Figure 13.1).

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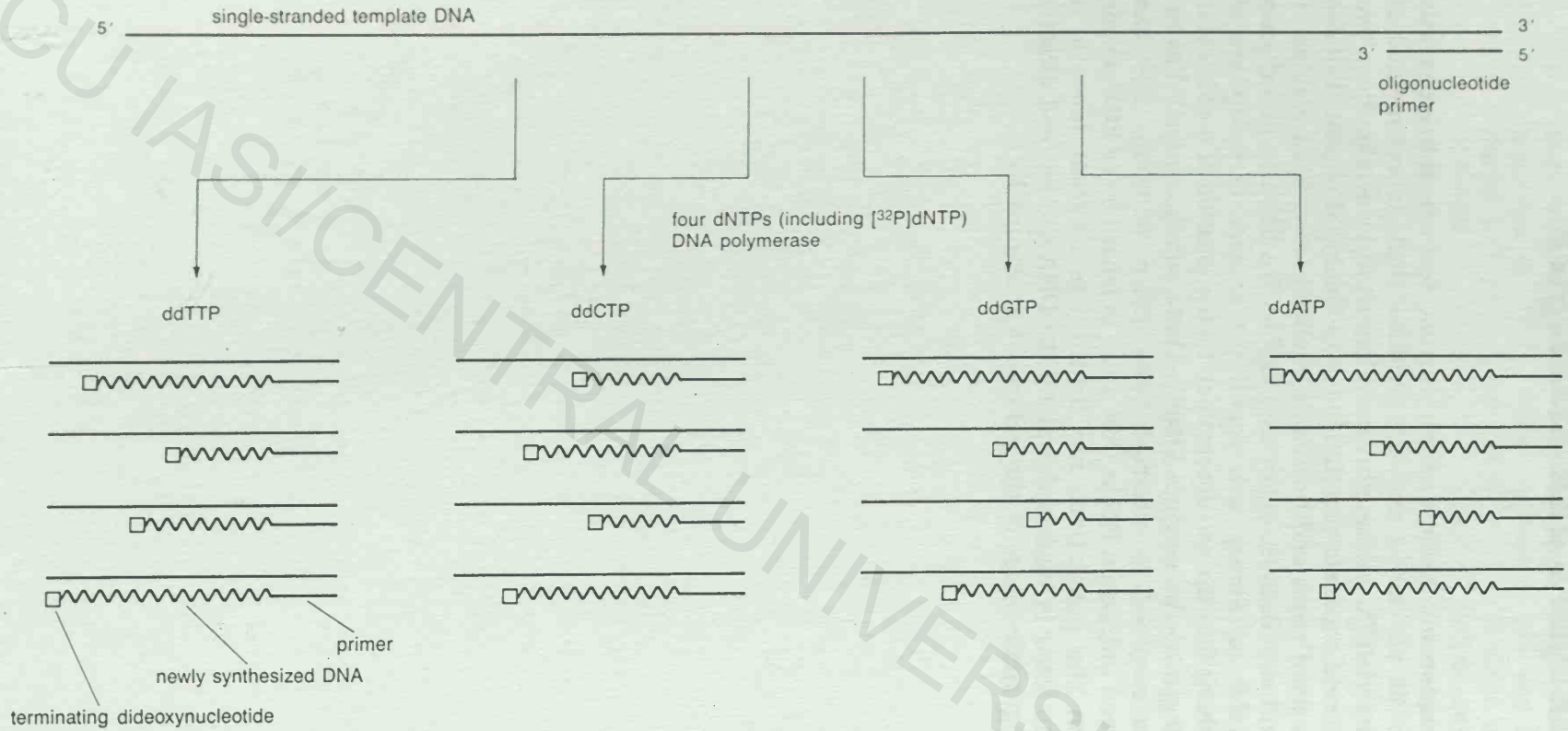

The newly synthesized chains terminate when a ddNTP is incorporated in place of the normal dNTP

Denature and separate fragments of radiolabeled DNA by electrophoresis

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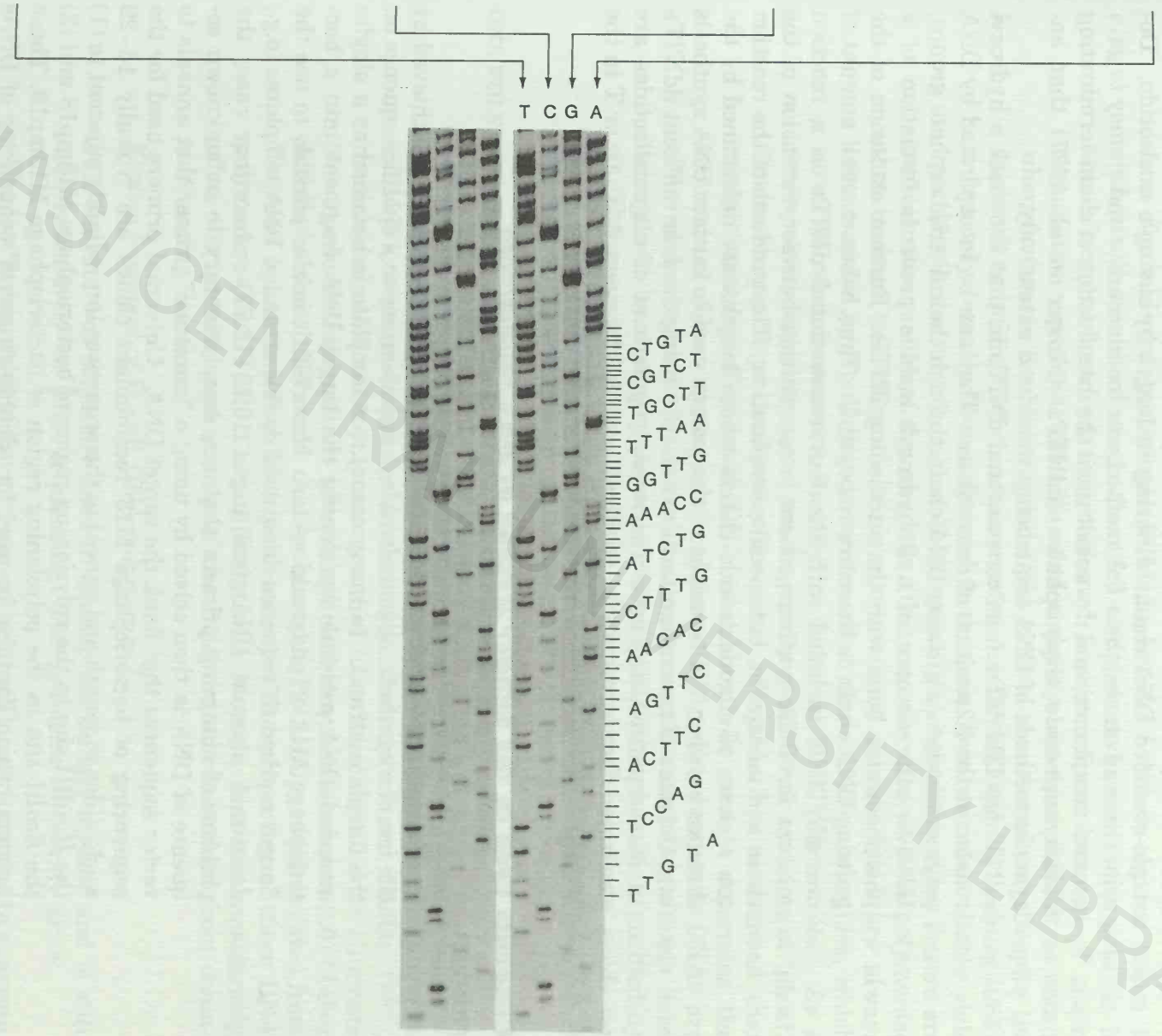

Sequencing by the Sanger dideoxy-mediated chain-termination method.

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SANGER METHOD OF DIDEOXY-MEDIATED CHAIN TERMINATION

The current chain-termination method evolved from the +/− sequencing technique (Sanger and Coulson 1975), which first described (1) the use of a specific primer for extension by DNA polymerase, (2) base-specific chain termination, and (3) the use of polyacrylamide gels to discriminate between single-stranded DNA chains differing in length by a single nucleotide. Despite these advances, the +/− method was too inaccurate and clumsy to gain general acceptance, and it was not until the introduction of chain-terminating dideoxynucleoside triphosphates (ddNTPs) (Sanger et al. 1977) that enzymatic methods of DNA sequencing were used extensively.

2′,3′ ddNTPs differ from conventional dNTPs in that they lack a hydroxyl residue at the 3′ position of deoxyribose. They can be incorporated by DNA polymerases into a growing DNA chain through their 5′ triphosphate groups. However, the absence of a 3′-hydroxyl residue prevents formation of a phosphodiester bond with the succeeding dNTP. Further extension of the growing DNA chain is therefore impossible. Thus, when a small amount of one ddNTP is included with the four conventional dNTPs in a reaction mixture for DNA synthesis, there is competition between extension of the chain and infrequent, but specific, termination. The products of the reaction are a series of oligonucleotide chains whose lengths are determined by the distance between the terminus of the primer used to initiate DNA synthesis and the sites of premature termination. By using the four different ddNTPs in four separate enzymatic reactions, populations of oligonucleotides are generated that terminate at positions occupied by every A, C, G, or T in the template strand (see Figure 13.1, pages 13.4–13.5).

Reagents Used in the Sanger Method of DNA Sequencing

PRIMERS

In enzymatic sequencing reactions, priming of DNA synthesis is achieved by the use of a synthetic oligonucleotide complementary to a specific sequence on the template strand. In many cases, this template is obtained as a single-stranded DNA molecule by cloning the target DNA fragment into a bacteriophage M13 or phagemid vector. However, it is also possible to use the Sanger method to sequence denatured double-stranded DNA templates (e.g., denatured plasmid DNA) (see pages 13.70–13.72). In either case, the problem of obtaining primers that are complementary to an unknown sequence of DNA is then solved by using a "universal" primer that anneals to vector sequences that flank the target DNA. Universal primers used for the sequencing of bacteriophage M13 recombinant clones are typically 15–29 nucleotides in length and anneal to the sequences immediately adjacent to (1) the *Hin*dIII site in the polycloning region of bacteriophage M13mp18 and (2) the *Eco*RI site in the polycloning region of bacteriophage M13mp19. These primers, which can also be used for "double-stranded" sequencing of DNAs cloned into pUC plasmids, are available from a large number of commercial suppliers. In addition, several companies sell primers that have been designed to allow sequencing of target DNAs cloned into a variety of restriction sites in different plasmids.

TEMPLATES

As mentioned above, two types of DNA can be used as templates in the Sanger method of sequencing: pure single-stranded DNA and double-stranded DNA that has been denatured by heat or alkali. The best results are obtained from single-stranded DNA templates, which are usually isolated from recombinant bacteriophage M13 particles. When care is taken to optimize the ratio of single-stranded template to primer, it is possible to obtain several hundred nucleotides of sequence from each set of chain-termination reactions. Results of this quality are more difficult to obtain when denatured double-stranded DNA is used as a template. Despite the apparent simplicity and convenience of the method (Chen and Seeburg 1985), it has only recently been improved to the point where it can reliably yield unambiguous results from double-stranded DNA templates. Two factors are critical: the quality of the template DNA and the type of DNA polymerase that is used (see below). Minipreparations of plasmid DNA are always contaminated by small oligodeoxyribonucleotides and ribonucleotides, which serve as random primers, and by inhibitors of DNA polymerases. As a consequence, sequencing gels are frequently obscured by a variety of "ghost" bands, strong stops, and other artifacts. We therefore recommend that minipreparations of plasmid DNAs should not be used to determine the sequence of cloned segments of unknown DNA. However, such DNAs are often adequate templates for confirming a sequence that has already been determined by another method. Plasmid DNA that has been purified by equilibrium centrifugation in CsCl–ethidium bromide gradients yields far better results, although the labor and expense of preparing plasmid DNA in this way are considerable.

DNA POLYMERASES

Several different enzymes are commonly used for dideoxy-mediated sequencing, including the Klenow fragment of *E. coli* DNA polymerase I (Sanger et al. 1977), reverse transcriptase (see, e.g., Mierendorf and Pfeffer 1987), bacteriophage T7 DNA polymerases that have been modified to eliminate $3' \rightarrow 5'$ exonuclease activity (Sequenase and Sequenase version 2.0) (Tabor and Richardson 1987), and the thermostable DNA polymerase isolated from *Thermus aquaticus* (*Taq* DNA polymerase). The properties of these DNA polymerases (see Table 13.1) differ greatly in ways that can considerably affect the quantity and quality of the DNA sequence obtained from chain-termination reactions.

Klenow fragment of E. coli *DNA polymerase I*

This enzyme was originally used to develop the Sanger method and is still used extensively for DNA sequencing. Two problems frequently arise:

- The low processivity of the enzyme causes the Klenow fragment to generate a high background of fragments that terminate, not by incorporation of a ddNTP, but by the random dissociation of the polymerase from the template. The inability of the enzyme to travel long distances along the template limits the length of sequence that can be obtained from standard

sequencing reactions using this enzyme. Typically, such reactions generate approximately 250 to 350 nucleotides of sequence. The amount of sequence can be doubled by carrying out the reaction in two steps—an initial labeling step containing low concentrations of dNTPs, followed by a chain-extension/chain-termination reaction containing ddNTPs and a high concentration of dNTPs (Johnston-Dow et al. 1987; Stambaugh and Blakesley 1988). However, even with these improvements, the Klenow enzyme does not routinely yield as much sequence as the more processive Sequenase enzymes (see below).

- The enzyme will not efficiently copy homopolymeric tracts or other regions of high secondary structure in the template. This problem can be alleviated, but not completely solved, by increasing the temperature of the polymerization reaction to 55°C (Gomer and Firtel 1985). dNTP analogs (e.g., dITP or 7-deaza-dGTP [see page 13.10]), which are sometimes used to obtain sequence information at regions of the template that form stable secondary structures, are less effective with the Klenow enzyme than with Sequenases, perhaps because they decrease still further the already low processivity of the enzyme.

In summary, the Klenow fragment of *E. coli* DNA polymerase I is the enzyme of choice for determining the sequence of tracts of DNA that lie within 250 bases of the 5′ terminus of the primer. It is not recommended for sequencing longer segments of DNA or DNAs with dyad symmetry and/or homopolymeric tracts.

Reverse transcriptase

Although not widely used for routine sequencing, this enzyme is occasionally employed to resolve problems caused by the presence of homopolymeric regions of A/T or G/C in the template DNA. Reverse transcriptases from both avian and murine sources appear to be slightly better in this respect than the Klenow enzyme (Karanthanasis 1982; Graham et al. 1986), although perhaps not as good as the Sequenases (Cameron-Mills 1988; Revak et al. 1988).

TABLE 13.1 Properties of DNA Polymerases Used in DNA Sequencing Reactions

Enzyme	Processivity[a]	Rate of polymerization[b]
Klenow fragment of *E. coli* DNA polymerase I	10–50	45
Reverse transcriptase (AMV)	n.d.	5
Sequenase and	~2000	
Sequenase version 2.0	~3000	300
Taq DNA polymerase and AmpliTaq[c]	>7600	35–100

[a] Processivity is expressed as the average number of nucleotides synthesized before the enzyme dissociates from the template; n.d. indicates not determined.
[b] Rate of polymerization in nucleotides/second.
[c] *Taq* DNA polymerase, a highly processive DNA polymerase, is useful for determining the sequence of DNA templates that form stable secondary structures.

Sequenases

Sequenase™ is a form of bacteriophage T7 DNA polymerase that has been chemically modified to eliminate much of the enzyme's powerful $3' \rightarrow 5'$ exonuclease activity. Sequenase version 2.0 is a genetically engineered form of Sequenase that entirely lacks $3' \rightarrow 5'$ exonuclease activity, is extremely stable, and has a threefold higher specific activity than the chemically modified enzyme. Sequenases are the enzymes of choice for determining the sequences of long tracts of DNA because of their very high processivity, their high rate of polymerization, and their wide tolerance for nucleotide analogs such as dITP and 7-deaza-dGTP that are used to resolve regions of compression in sequencing gels. Sequenases travel such long distances along the template that several hundred nucleotides of DNA sequence can often be determined from a single set of reactions. In fact, the amount of sequence is limited more by the resolving power of polyacrylamide gels than by the properties of the enzyme.

To take full advantage of the high processivity of Sequenases, a two-step sequencing reaction is set up. In the first stage, low concentrations of dNTPs and low temperature are used to limit the extent of synthesis and to ensure efficient incorporation of a radiolabeled dNTP. The products of this reaction are primers that have been extended by only 20–30 bases. The first reaction is then divided into the standard set of four reactions, each of which contains high concentrations of dNTPs and a single ddNTP. Polymerization then continues until a chain-terminating nucleotide is incorporated into the growing chain.

Taq *DNA polymerase*

Taq DNA polymerase is useful for determining the sequence of single-stranded DNA templates that form extensive stable secondary structures at 37°C. This is because *Taq* DNA polymerase works efficiently at 70–75°C, a temperature that precludes formation of secondary structure even in templates that are rich in G + C. When used as described by Innis et al. (1988), sequencing ladders produced by *Taq* DNA polymerase demonstrate a high uniformity of band intensity for several hundred nucleotides, suggesting that the enzyme has a high degree of processivity.

RADIOLABELED dNTPs

Until a few years ago, virtually all DNA sequencing was carried out with [α-^{32}P]dNTPs. However, the strong β particles emitted by ^{32}P created two problems. First, because of scattering, the bands on the autoradiograph were far larger and more diffuse than the bands of DNA in the gel. This affected the ability to read a sequence correctly (particularly from the upper part of the autoradiograph) and limited the number of nucleotides that could be read from a single gel. Second, decay of ^{32}P caused radiolysis of the DNA in the sample. Sequencing reactions radiolabeled with ^{32}P could therefore be stored for only 1 or 2 days before the DNA was so badly damaged that it generated indecipherable sequencing gels.

The introduction of [^{35}S]dATP (Biggin et al. 1983) greatly alleviated both of these problems. Because of the decreased scatter of the weaker β particles

produced by decay of ^{35}S, there is little loss of resolution between the gel and the autoradiograph. This allows unambiguous determination of several hundred nucleotides of DNA sequence from a single reaction set. Furthermore, the lower energy of ^{35}S produces less radiolysis, allowing sequencing reactions to be stored for up to 1 week at −20°C without noticeable loss of resolution. Thus, if technical problems arise with a polyacrylamide gel, the sequencing reactions can simply be reanalyzed.

ANALOGS OF dNTPs

Regions of DNA with dyad symmetry (especially those with a high G + C content) can form intrastrand secondary structures that are not fully denatured during electrophoresis. This can cause an anomalous pattern of migration in which adjacent bands of DNA become compressed to the point where they are difficult to read. Compression is entirely dependent on the presence of secondary structures in DNA and cannot be alleviated by changing the type of DNA polymerase used in the sequencing reaction. However, compressed regions of gels can usually be resolved by using a nucleotide analog such as dITP (2′-deoxyinosine-5′-triphosphate) or 7-deaza-dGTP (7-deaza-2′-deoxyguanosine-5′-triphosphate). These analogs pair weakly with conventional bases and are good substrates for DNA polymerases such as the Sequenases and *Taq* DNA polymerase (Gough and Murray 1983; Mizusawa et al. 1986; Innis et al. 1988). Some compressions are not resolved by 7-deaza-dGTP; others (particularly those occurring in GC-rich regions) are not resolved by dITP. If it is necessary to use analogs, try dITP first (see pages 13.74–13.75). This analog, in contrast to 7-deaza-dGTP, does not affect the sharpness of the DNA bands in the sequencing gel. Any compression that is not resolved by either dITP or 7-deaza-dGTP can almost always be cleared up by determining the sequence of both strands of the DNA.

As discussed above, both forms of Sequenase and *Taq* DNA polymerase tolerate nucleotide analogs better than does the Klenow fragment of *E. coli* DNA polymerase I. In addition, the manufacturer claims that Sequenase version 2.0 is superior to the original enzyme when sequencing templates with strong secondary structure. Version 2.0 is more processive than Sequenase, having less tendency to pause, thereby eliminating "ghost" bands. Furthermore, version 2.0 appears to tolerate nucleotide analogs such as dITP better than does the original version.

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MAXAM-GILBERT CHEMICAL DEGRADATION OF DNA METHOD

Unlike the chain-termination technique, which involves synthesis, the Maxam-Gilbert method involves chemical degradation of the original DNA. This method grew out of studies of the interaction between the *lac* repressor and the *lac* operator in vitro. To this day, the ability to probe DNA conformations and protein–DNA interactions remains a unique feature of the Maxam-Gilbert method.

In this procedure (Maxam and Gilbert 1980), a fragment of DNA radiolabeled at one end is partially cleaved in five separate chemical reactions, each of which is specific for a particular base or type of base. This generates five populations of radiolabeled molecules that extend from a common point (the radiolabeled terminus) to the site of chemical cleavage. Each population consists of a mixture of molecules whose lengths are determined by the locations of a particular base along the length of the original DNA. These populations are then resolved by electrophoresis through polyacrylamide gels, and the end-labeled molecules are detected by autoradiography (see Figure 13.2).

The Maxam-Gilbert method has remained relatively unchanged since its initial development. Although additional chemical cleavage reactions have been devised (for review, see Ambrose and Pless 1987), these are generally used to supplement the reactions originally described by Maxam and Gilbert (1977, 1980). The success of the method depends entirely on the specificity of these cleavage reactions, which are carried out in two stages. In the first stage, specific bases (or types of bases) undergo chemical modification; in the second stage, the modified base is removed from its sugar and the phosphodiester bonds 5′ and 3′ to the modified base are cleaved (see Table 13.2). In every case, these reactions are carried out under carefully controlled conditions to ensure that on average only one of the target bases in each DNA molecule is modified. Subsequent cleavage by piperidine at the 5′ and 3′ sides of the modified bases yields a set of end-labeled molecules whose lengths range from one to several hundred nucleotides. The DNA sequence can then be read from an autoradiograph of a sequencing gel by comparing the G, A + G, C + T, C, and A > C tracks. For a number of reasons (e.g., the use of ^{32}P as a radiolabel, the specific activity of end-labeled DNA, the statistical distribution of cleavage sites, and the limitations of gel technology), the range of the Maxam-Gilbert method is less than that of the Sanger method; the Maxam-Gilbert method works optimally for DNA sequences that lie less than 250 nucleotides from the radiolabeled end.

When the Maxam-Gilbert and Sanger methods were first developed in the 1970s, sequencing by chemical degradation was both more reproducible and more accessible to the average worker. The Sanger method required single-stranded templates, specific oligonucleotide primers, and access to high-quality preparations of the Klenow fragment of *E. coli* DNA polymerase I. The Maxam-Gilbert method used simple chemical reagents that were available to everyone. However, with the development of bacteriophage M13 and phagemid vectors, the ready availability of synthetic primers, and improvements to the sequencing reactions, the dideoxy-mediated chain-termination method is now used much more extensively than the Maxam-Gilbert method. Nevertheless, the chemical degradation approach has one clear advantage

over the chain-termination method: Sequence is obtained from the original DNA molecule and not from an enzymatic copy. Therefore, with the Maxam-Gilbert method, one can sequence synthetic oligonucleotides, analyze DNA modifications such as methylation, and study both DNA secondary structure and the interaction of proteins with DNA by either chemical protection or modification interference experiments. However, because of its ease and rapidity, the Sanger technique is now the best choice for simple determination of DNA sequence. In fact, most of the current sequencing strategies have been designed for use with this method.

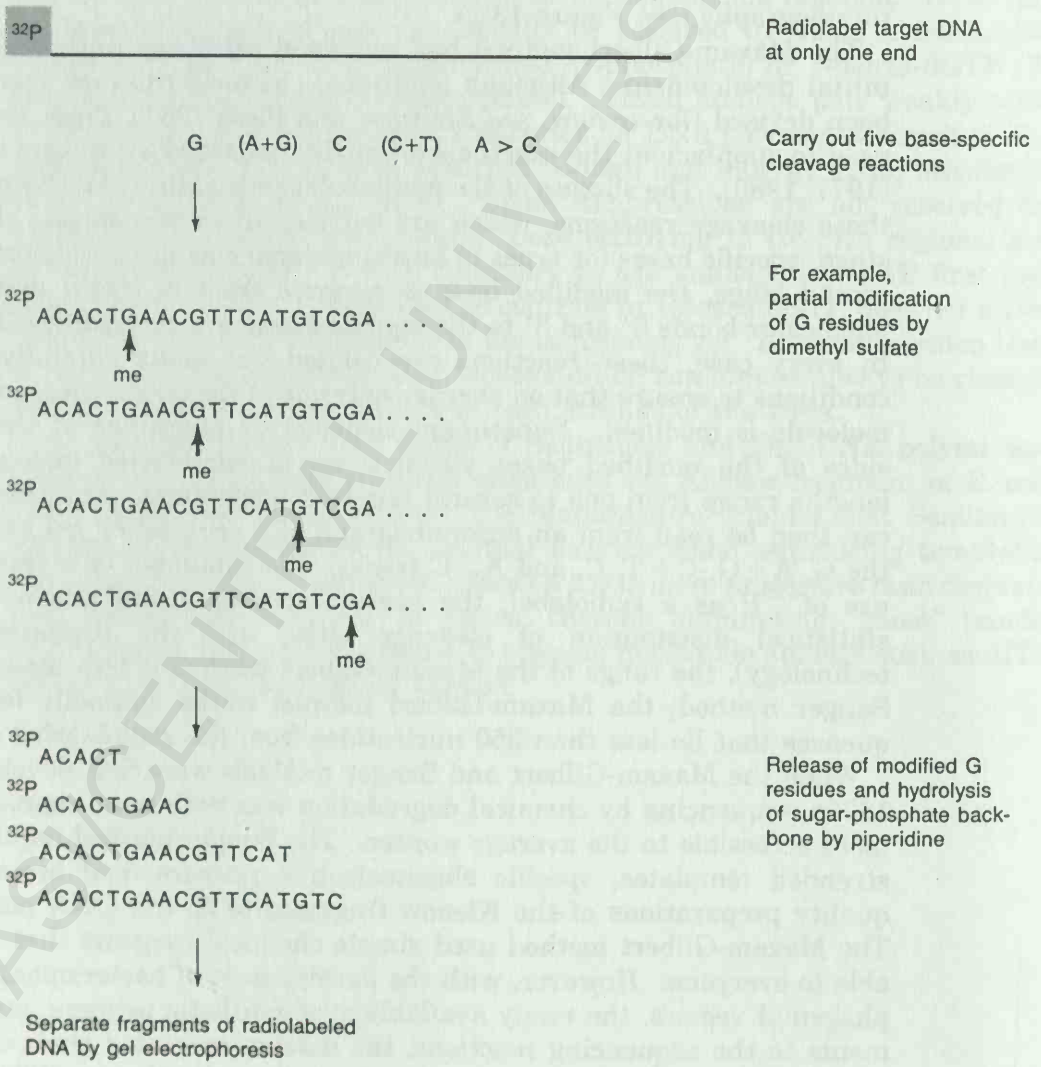
32P
Radiolabel target DNA at only one end
G (A+G) C (C+T) A > C
Carry out five base-specific cleavage reactions
32P ACACTGAACGTTCATGTCGA . . . .
me
For example, partial modification of G residues by dimethyl sulfate
32P ACACTGAACGTTCATGTCGA . . . .
me
32P ACACTGAACGTTCATGTCGA . . . .
me
32P ACACTGAACGTTCATGTCGA . . . .
me
32P ACACT
32P ACACTGAAC
32P ACACTGAACGTTCAT
32P ACACTGAACGTTCATGTC
Release of modified G residues and hydrolysis of sugar-phosphate backbone by piperidine
Separate fragments of radiolabeled DNA by gel electrophoresis

FIGURE 13.2
Sequencing by the Maxam-Gilbert chemical degradation of DNA method.

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TABLE 13.2 Chemical Modifications Used in the Maxam-Gilbert Method

Base	Specific modification[a]
G	Methylation of N_7 with dimethyl sulfate at pH 8.0 makes the C_8—C_9 bond specifically susceptible to cleavage by base
A + G	Piperidine formate at pH 2.0 weakens the glycosidic bond of adenine and guanine by protonating nitrogen atoms in the purine rings resulting in depurination
C + T	Hydrazine opens pyrimidine rings, which recyclize in a five-membered form that is susceptible to removal
C	In the presence of 1.5 M NaCl, only cytosine reacts appreciably with hydrazine
A > C	1.2 N NaOH at 90°C results in strong cleavage at A and weaker cleavage at C

[a] Hot (90°C) piperidine (1 M in H_2O) is used to cleave the sugar-phosphate chain of DNA at the sites of chemical modifications.

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SEQUENCING STRATEGIES

Before beginning to sequence, it is important to develop an overall strategy that takes into account the size of the region to be sequenced, the accuracy of the sequence required, and the facilities that are available. Only a minor proportion of projects involve the de novo accumulation of large tracts of virgin sequence. More often, sequencing is used to map and identify mutations (e.g., point mutations and deletions) and to verify the orientation and structure of recombinant DNA constructs. The approaches used for these two purposes are very different.

Confirmatory Sequencing

Confirmatory sequencing (e.g., sequencing of mutants generated by oligonucleotide-mediated mutagenesis) often requires no more than one set of reactions that generates the nucleotide sequence of a local region of one of the two strands of DNA. This can usually be achieved by sequencing an appropriate restriction fragment that has been subcloned into a bacteriophage M13 or phagemid vector. In many cases, the region of interest will lie within the sequencing range of a universal primer; if not, the best strategy is to synthesize a priming oligonucleotide 17–19 nucleotides long that is complementary to sequences located approximately 50–100 nucleotides from the region of interest. Whenever possible, the sequence of the homologous region of the wild-type gene should be determined at the same time as that of the mutant. Direct comparison of the sequences on the same autoradiograph greatly facilitates confirmation of the sequence of the altered region and clearly reveals any unexpected, additional differences between mutant and wild-type genes.

De Novo Sequencing

The aim of de novo sequencing is to provide the accurate nucleotide sequence of a virgin segment of DNA that may be many kilobases in length. This task requires careful planning because the maximum length of target DNA that can be sequenced accurately in a single set of sequencing reactions is approximately 400 bases. Target DNAs of this size can be sequenced by cloning in opposite orientations in each of two bacteriophage M13 vectors (e.g., M13mp18 and M13mp19). The entire sequence of each strand can then be determined in a single reaction set using a universal sequencing primer. To sequence longer target DNAs (e.g., several kilobases in length), one of two general strategies can be used:

- *A random approach (or shotgun sequencing),* in which sequence data are collected from subclones containing random segments of the target DNA. No attempt is made to determine where these subclones map in the target DNA or which strand of DNA is being sequenced. Instead, the accumulated data are stored and finally arranged in order by a computer (Staden 1986). This approach, which was pioneered by the M.R.C. Laboratory in Cambridge, has been used successfully to determine the sequences of human mitochondrial DNA (Anderson et al. 1981), human adenovirus DNA (Gin-

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geras et al. 1982; Roberts et al. 1986), bacteriophage λ DNA (Sanger et al. 1982), and Epstein-Barr virus DNA (Baer et al. 1984).

- *Directed approaches,* in which sequences of the target DNA are obtained in a systematic fashion. For example, the entire sequence of the target DNA might be obtained by sequencing a nested set of deletion mutants that begin at a common point (usually at one end of the target DNA) and penetrate various distances into the target region. They therefore bring progressively more remote regions of the target DNA into range for sequencing by universal primer (see Figure 13.3A). Alternatively, virgin segments of target DNA can be sequenced in a step-wise fashion by using the nucleotide sequences obtained from one set of reactions to design a new oligonucleotide that is then used to prime the subsequent set of reactions. In this approach, therefore, DNA sequence is accumulated by moving the priming site along the DNA in a progressive fashion (see Figure 13.3B).

Although the choice between the random and directed strategies is usually dictated by the resources and expertise that are available in the laboratory, a number of additional factors that may also influence the final selection are discussed on pages 13.18–13.20.

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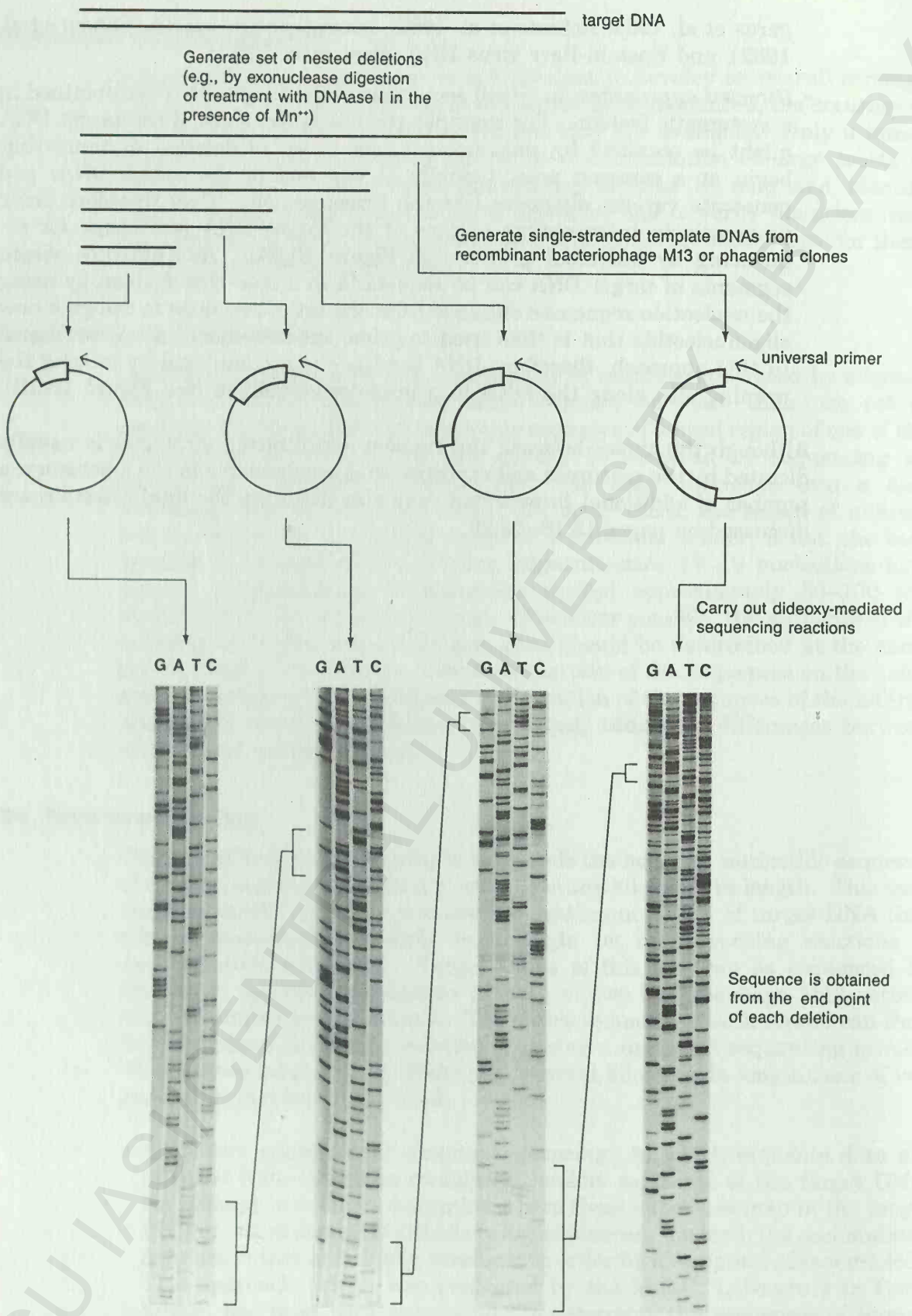
target DNA
Generate set of nested deletions (e.g., by exonuclease digestion or treatment with DNAase I in the presence of Mn++)
Generate single-stranded template DNAs from recombinant bacteriophage M13 or phagemid clones
universal primer
Carry out dideoxy-mediated sequencing reactions
G A T C
G A T C
G A T C
G A T C
Sequence is obtained from the end point of each deletion

FIGURE 13.3A
Directed sequencing with nested sets of deletion mutants.

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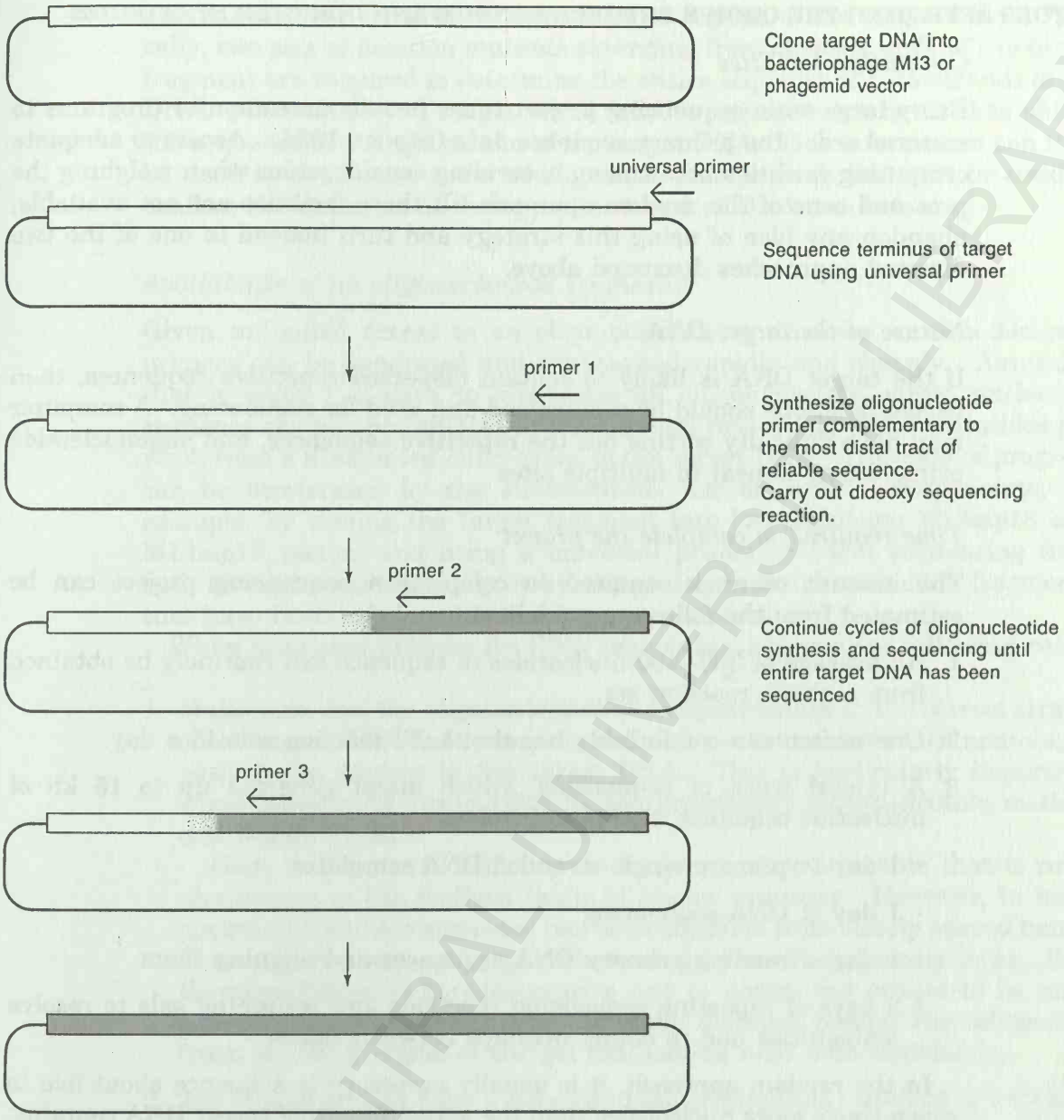
Clone target DNA into bacteriophage M13 or phagemid vector
universal primer
Sequence terminus of target DNA using universal primer
primer 1
Synthesize oligonucleotide primer complementary to the most distal tract of reliable sequence.
Carry out dideoxy sequencing reaction.
primer 2
Continue cycles of oligonucleotide synthesis and sequencing until entire target DNA has been sequenced
primer 3

FIGURE 13.3B
Directed sequencing with progressive oligonucleotides.

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Computing facilities

Every large-scale sequencing project relies heavily on computer programs to sort and order the primary sequence data (Staden 1986). Access to adequate computing facilities becomes an overriding consideration when weighing the pros and cons of the random approach. If these facilities are not available, abandon any idea of using this strategy and turn instead to one of the two directed approaches discussed above.

Nature of the target DNA

If the target DNA is likely to contain dispersed repetitive sequences, then nested deletions should be constructed and used for sequencing. A computer may have difficulty sorting out the repetitive sequences, and oligonucleotide primers may anneal to multiple sites.

Time required to complete the project

The amount of work required to complete a sequencing project can be estimated from the following guidelines:

1. An average of 300–400 nucleotides of sequence can routinely be obtained from a single reaction set.
2. One person can comfortably handle 24–32 reaction sets in a day.
3. A typical week of sequencing, which might generate up to 15 kb of nucleotide sequence, therefore involves
   - 1 day to prepare single-stranded DNA templates
   - 1 day of DNA sequencing
   - 1 day of reading primary DNA sequences and aligning them
   - 2 days of repeating sequencing reactions and rerunning gels to resolve ambiguities and to obtain overlaps between clones

In the random approach, it is usually necessary to sequence about five to seven times more nucleotides than the actual length of target DNA contains. In most cases, a single contiguous sequence does not emerge until approximately 90% of the sequence of both strands has been determined. Because subclones for sequencing are selected at random, certain regions of the target DNA will be sequenced repeatedly before the entire region is covered, and there is no way to predict how long it will take to find and sequence the last subclones required to complete a sequence. Often, these subclones turn out to be underrepresented in the library, and it is then necessary to isolate them by screening with oligonucleotide probes corresponding to flanking sequences. These logistic problems can be alleviated by using restriction enzymes to subdivide large target DNAs into pieces of manageable size (4–5 kb). Each of these pieces is then sequenced independently by the random method.

The directed deletion approach sometimes requires a considerable investment of time to generate and characterize a complete set of nested deletions. However, once this step has been accomplished, informative DNA sequence is

accumulated simultaneously from ordered regions of the target DNA. Typically, two sets of deletion mutants extending from opposite ends of the target fragment are required to determine the entire sequence of both strands of the DNA. Alternatively, a single set of deletion mutants can be used to obtain the sequence of a single strand of the target DNA. This information can then be used to synthesize a set of oligonucleotide primers that can be used to confirm the sequence of the opposite strand of DNA (see below).

Availability of an oligonucleotide synthesizer

Given unlimited access to an oligonucleotide synthesizer, custom-designed primers can be generated and synthesized rapidly and cheaply. Assuming that it takes 1–2 days to synthesize an oligonucleotide, virgin nucleotide sequence can be accumulated at a maximum rate of 600–800 nucleotides per week from a designated entry point to the target DNA. This rate of progress can be accelerated by the simultaneous use of several entry points, for example, by cloning the target fragment into bacteriophage M13mp18 and M13mp19 vectors and using a universal primer to begin sequencing from both termini simultaneously or by sequencing internal restriction fragments that have been subcloned into appropriate vectors.

When designing primers for DNA sequencing, observe the following rules:

1. Make sure that the oligonucleotide is complementary to the correct strand of the target DNA and to sequences that have been determined unambiguously to be present in the target DNA. This is particularly important when sequencing virgin DNA by the progressive oligonucleotide method (see Figure 13.3B).

   There is a natural tendency to design an oligonucleotide that is complementary to the furthest limits of known sequence. However, in most circumstances, this sequence has been obtained from closely spaced bands at the top of a sequencing gel, where reading errors frequently occur. It is therefore better to be conservative and to design the primer to be complementary to sequences that lie some distance behind the advancing front, within a region of the gel that can be read with confidence.

2. The primer should have a balanced base composition (40–55% G + C) and should be a least 18 nucleotides in length. If the %(G + C) lies outside these limits, design an oligonucleotide whose length is $18 + n/2$ nucleotides, where $n = 50 - \%(G + C)$ for A/T-rich regions and $n = \%(G + C) - 50$ for G/C-rich regions.

3. Check the sequence of the proposed primer to ensure that

   a. It does not contain regions of dyad symmetry. Oligonucleotides that can self-hybridize to form hairpin or stem-loop structures are inefficient primers.

   b. It is not complementary either to the vector DNA or to regions of the target DNA that have already been sequenced. This greatly reduces the possibility that the oligonucleotide might prime DNA synthesis from more than one location on the template DNA. Most commercially available computer programs for the analysis of DNA have the ability

to search sequences for regions that are complementary to synthetic oligonucleotides.

Accuracy of the sequence

When DNA sequencing is carried out carefully, the error rate is less than 0.1%. However, to achieve this high rate of accuracy, it is necessary to sequence both strands of the target DNA completely and to resolve all ambiguities and discrepancies. In this respect, random sequencing has an advantage, since the gradual accumulation of redundant primary sequences greatly improves the accuracy of the final assembled sequence. However, there may be regions of the target DNA that cannot be sequenced accurately by either the random method or directed methods. Resolving these difficult sequences often takes a surprisingly long time and sometimes requires the use of base analogs (to eliminate compressions) or Maxam-Gilbert sequencing.

Future direction of the project

Different sequencing strategies yield different types of material that can be used in later experiments. For example, nested sets of deletions generated for DNA sequencing can be used to study the domains within a promoter region or sets of oligonucleotides complementary to different regions of the target fragment can be used to sequence mutant forms of the target sequence. Random subclones created for shotgun sequencing provide a store of material that can subsequently be used for site-directed mutagenesis or for the generation of radiolabeled probes.

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Random Sequencing

Three methods can be used to break large target DNAs into segments that are optimal in size for random sequencing: digestion with restriction enzymes, sonication, and digestion with pancreatic DNAase I in the presence of Mn^{++}. Sonication and digestion with DNAase I are superior to digestion with restriction enzymes in a number respects. First, they cleave DNA in a random fashion that is relatively independent of sequence, and therefore potential difficulties caused by uneven distribution of restriction sites are avoided. Second, both methods yield a collection of overlapping fragments—something that is impossible when DNA is digested to completion with a single restriction enzyme. In theory, sets of overlapping fragments can be obtained by partial digestion of the target DNA with a cocktail containing several frequently cutting restriction enzymes. In practice, this turns out to be difficult to achieve because different restriction enzymes cleave at vastly different rates. In contrast, random fragmentation follows a predictable time course once the system has been calibrated. The only major disadvantage of fragmentation either by sonication or by digestion with DNAase I in the presence of Mn^{++} is that the resulting small pieces of DNA carry frayed ends that must be repaired before cloning into bacteriophage M13 or phagemid vectors. This step is inefficient and, in the past, it was sometimes difficult to obtain enough clones to saturate the target DNA. This problem was largely solved when bacteriophage T4 DNA polymerase of high quality became available from commercial sources. Typically, fragmented DNA that has been repaired with this enzyme yields between 500 and 1000 subclones per microgram of starting DNA. This is far in excess of the number of subclones required to assemble a complete sequence of a target DNA less than 10 kb in length. The steps involved in fragmentation, repair, and cloning are outlined below and illustrated in Figure 13.4.

1. *Purification of the target DNA.* The target DNA is separated from vector sequences by digestion with restriction enzymes and polyacrylamide or agarose gel electrophoresis. If the target DNA and the vector are similar in size and cannot be separated by gel electrophoresis, try to find a restriction enzyme that will cleave the vector into several pieces without digesting the target DNA. This alleviates the problem of sequencing clones that contain only fragments of the vector. Alternatively, the collection of recombinant clones can be screened by hybridization to radiolabeled DNA of the parental vector. Only those clones that fail to hybridize (i.e., which carry inserts consisting entirely of target DNA) are selected for sequencing. The sequences of the terminal regions of the target DNA that formed junctions with the original vector can be obtained by subcloning appropriate restriction fragments into bacteriophage M13 or phagemid vectors.
2. *Ligation.* The purified target DNA is ligated to itself, generating a series of concatenated and/or circular molecules that are then subjected to

fragmentation. This ligation step is important because it ensures that all segments of the target DNA have an equal chance of being fragmented by sonication. Without ligation, monomeric linear target DNA will tend to be sheared preferentially near its center during sonication.

The ligation step is less important if the DNA is fragmented by digestion with DNAase I in the presence of Mn^{++}. However, it is still worthwhile because it helps to ensure that all segments of the target DNA will be equally represented in the final library. Without ligation, the ends of the target DNA will be cloned preferentially and will be overrepresented in the resulting library.

3. *Fragmentation.* When fragmenting DNA for the first time, either by sonication or by digestion with DNAase I in the presence of Mn^{++}, it is

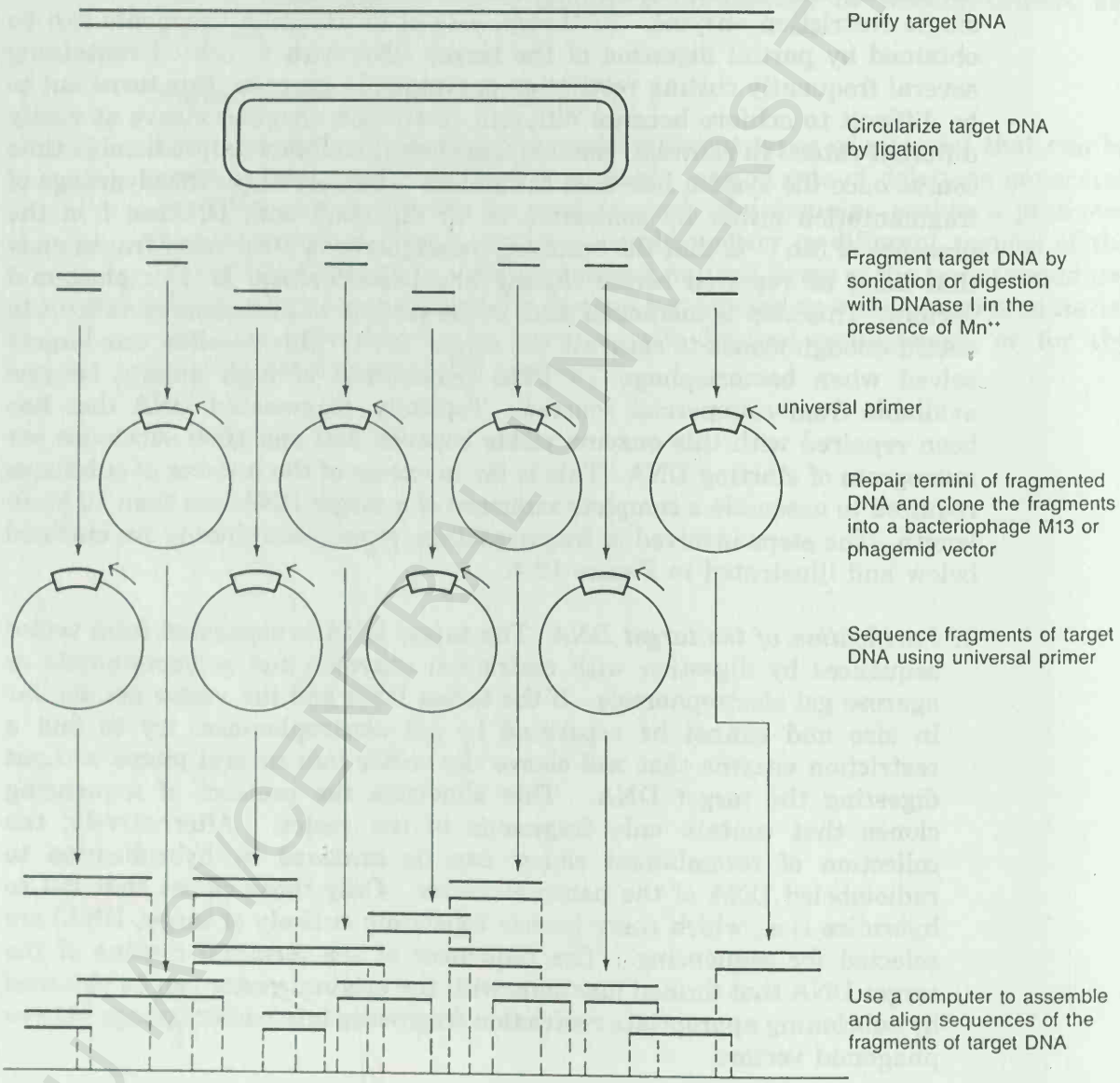
Purify target DNA
Circularize target DNA by ligation
Fragment target DNA by sonication or digestion with DNAase I in the presence of Mn++
universal primer
Repair termini of fragmented DNA and clone the fragments into a bacteriophage M13 or phagemid vector
Sequence fragments of target DNA using universal primer
Use a computer to assemble and align sequences of the fragments of target DNA

Figure 13.4
Random sequencing.

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essential to carry out a series of calibration experiments to establish conditions that yield acceptable quantities of fragments of the desired size. This is best done by performing a series of preliminary experiments with a control DNA of defined size, for example, bacteriophage λ DNA.

4. *Size selection, repair, and cloning.* DNA fragments of the desired size (500–800 bp) are isolated by gel electrophoresis and their termini are repaired with bacteriophage T4 DNA polymerase. The DNAs are then ligated to an appropriately prepared vector (e.g., bacteriophage M13mp18 or M13mp19 that has been linearized with a restriction enzyme that generates blunt ends). Finally, the ligated DNA is transfected into a suitable strain of *E. coli* (see Chapter 4), and single-stranded DNAs prepared from plaque-purified bacteriophages are used as templates in the Sanger dideoxy-mediated chain-termination method of sequencing.

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GENERATION OF A LIBRARY OF RANDOMLY OVERLAPPING CLONES

Purification and Ligation of the Target DNA

1. Using the appropriate restriction enzymes, digest a sufficient amount of recombinant vector to yield 10–15 μg of purified target DNA. Separate the target DNA from the vector by preparative gel electrophoresis, and recover the target DNA from the gel by one of the methods described in Chapter 6. Redissolve the purified DNA in 15 μl of TE (pH 7.6). Check the integrity and recovery of the purified DNA by analyzing an aliquot (0.5 μl) by agarose gel electrophoresis.

   Because the next step of the procedure requires the target DNA to be ligated to itself, it is best to use restriction enzymes that generate compatible termini. If this is not possible, an acceptable alternative is to use restriction enzymes that generate incompatible protruding termini and then to alter the ligation conditions as described in the note to step 2. If it is not possible to avoid restriction enzymes that generate blunt ends, the ligation (step 2) should be carried out in the presence of substances that increase macromolecular crowding (e.g., polyethylene glycol [Pheiffer and Zimmerman 1983]; see Chapter 1, pages 1.70–1.71).

2. Transfer 5–10 μg of the purified DNA to a fresh microfuge tube. Add:

10 × bacteriophage T4 DNA ligase buffer	2.5 μl
5 mM rATP	2.5 μl
30% w/v polyethylene glycol (PEG 8000) (optional—see note to step 1)	5.0 μl
H_2O to 25 μl	
bacteriophage T4 DNA ligase	
for cohesive termini	1.0 Weiss unit
for blunt termini	5.0 Weiss units

   Incubate the mixture at 16°C for 4 hours, and then inactivate the ligase by heating the mixture to 68°C for 15 minutes.

10 × Bacteriophage T4 DNA ligase buffer

200 mM Tris · Cl (pH 7.6)
50 mM $MgCl_2$
50 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

This buffer should be stored in small aliquots at −20°C.

If the termini of the purified target DNA are compatible, the products of ligation will be a series of oligomeric linear and circular DNA molecules in which adjacent segments of target DNA are arranged as either direct or inverted repeats. Up to 50% of the sonicated fragments carrying junction sequences will contain inverted repetitions and will be difficult to clone in bacteriophage M13 vectors. This problem becomes much worse if the termini of the purified target DNA are incompatible. In this case, the major products of ligation will be circular DNA molecules containing only inverted repetitions of the target DNA, and virtually all of the sonicated fragments containing junction sequences will carry inverted repetitions. To solve this problem, ligate only two thirds of the original target DNA and mix the products of ligation with the remaining one third of the unligated DNA before proceeding to the sonication step.

Several different assays have been used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units (as defined by New England Biolabs). 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units.

3. Add 175 μl of TE (pH 7.6), and purify the ligated DNA by extraction with phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube. Add 50 μl of 10 M ammonium acetate and 2 volumes of ethanol at room temperature. Store the microfuge tube on ice for 10 minutes, and then collect the precipitate of DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge. Remove the supernatant, wash the pellet with 0.5 ml of 70% ethanol at room temperature, and recentrifuge. Remove as much as possible of the supernatant, and allow the last traces of ethanol to evaporate at room temperature. Redissolve the DNA in 25–50 μl of TE (pH 7.6) to a final concentration of 200 μg/ml.

4. Fragment the DNA either by sonication or by digestion with DNAase I in the presence of Mn^{++} (see pages 13.26–13.29).

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Fragmentation of the Target DNA

As discussed above (see page 13.21), two methods—sonication and digestion with DNAase I in the presence of Mn^{++}—are commonly used to fragment the target DNA randomly into pieces whose sizes are convenient for sequencing in bacteriophage M13 or phagemid vectors. If a suitable sonicator is available, sonication is the method of choice. Once the sonicator is calibrated, fragmentation proceeds at a predictable rate that varies little from preparation to preparation of target DNA. On the other hand, the rate of fragmentation with DNAase I in the presence of Mn^{++} is more variable, often proceeds asynchronously, and is sensitive to trace contaminants in the DNA (e.g., sulfated polysaccharides from agarose gels).

SONICATION

The following method is a modification of a procedure described by Bankier et al. (1987). In almost all cases, the segment of DNA that is to be sequenced will already have been cloned and propagated in a plasmid, cosmid, or bacteriophage λ vector. To avoid generating subclones that carry vector sequences, it is necessary to recover the target DNA from the parental clone.

1. Calibrate the sonication apparatus. The times of sonication given below are for a cup-horn sonicator with a nominal peak output energy of 475 watts. However, because the actual output of different sonicators varies widely, it is necessary to calibrate each instrument as follows:

   a. Transfer 25 μl of a solution containing 200 μg/ml of bacteriophage λ DNA (or some other large DNA of defined molecular weight) to a microfuge tube.

   b. Fill the cup horn of the sonicator with ice water, and clamp the microfuge tube containing the DNA just above the probe.

   c. Sonicate at maximum power for a total of four 30–40 second bursts. After each burst, transfer 3 μl of the DNA to a fresh microfuge tube. Store this tube on ice. Between bursts, centrifuge the original microfuge tube for 2 seconds to deposit the DNA solution at the bottom of the tube, and add fresh ice to the sonicator horn to maintain the temperature of the DNA solution at 0°C.

   e. After sonication is completed, analyze the size of the DNA fragments in each sample stored in step c by electrophoresis through a 1.4% agarose gel. Use as marker pUC18 or pUC19 DNA that has been cleaved to completion with *Sau*3AI (see Appendix E).

   f. Stain and photograph the gel, and determine the amount of sonication required to produce a reasonable yield of fragments of the desired size (300 bp–1 kb).

By far the best type of apparatus is a cup-horn sonicator (e.g., W-385 Sonicator from Heat Systems Ultrasonics) in which the DNA samples, dissolved in small volumes of buffer, are enclosed in microfuge tubes and do not come into contact with the probe. If

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a cup-horn sonicator is not available, a conventional sonicator equipped with a microtip probe can be used. However, in this case, it is essential to remove contaminating DNA by cleaning the probe fastidiously before and after it is immersed in each sample. This is best done by boiling the sonicator probe in 1% SDS, 0.001 M EDTA for 10 minutes and then rinsing it thoroughly in sterile water. In addition, the volume of the DNA sample must be increased to at least 250 μl to accommodate the probe. After sonication is completed, the DNA is reconcentrated by precipitation with 2 volumes of ethanol and redissolved in 25 μl of TE (pH 7.6).

2. Sonicate the purified, ligated target DNA (from step 3, page 13.25) for exactly the same length of time calculated to produce a reasonable yield of fragments of the desired size (300 bp–1 kb).

   DNA that has been sonicated for excessive periods of time is extremely difficult to clone. Most sonicators will not shear DNA to a size smaller than 300—500 bp, and it is tempting to continue sonication until the entire population of DNA fragments has been reduced to this size. However, the yield of subclones is usually greater if sonication is stopped when the fragments of target DNA first reach a size of 300 bp–1 kb.

3. Proceed with repair and size selection of DNA as described on page 13.30.

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DIGESTION WITH DNAase I IN THE PRESENCE OF MANGANESE IONS

Pancreatic DNAase I normally introduces single-strand nicks into double-stranded DNA. In the presence of Mn^{++}, however, the enzyme cleaves both strands of DNA (Melgar and Goldthwait 1968) to generate molecules that are blunt-ended or have protruding termini only 1 or 2 nucleotides in length. Before the following protocol is used to fragment valuable target DNA, it should be tested with bacteriophage λ DNA or another large DNA of defined molecular weight.

1. Make up stock solution of DNAase I (1 mg/ml in 0.01 N HCl). Divide the enzyme solution into small aliquots (10 μl), and store them in small microfuge tubes at −70°C.

2. When you are ready to start the digestion, remove an aliquot of DNAase I from storage at −70°C and dilute it 1:1000 into ice-cold 1× DNAase/Mn^{++} digestion buffer. The concentration of the diluted enzyme should be 1 ng/μl.

10× DNAase/Mn^{++} digestion buffer

500 mM Tris · Cl (pH 7.6)
100 mM $MnCl_2$
1 mg/ml bovine serum albumin (Fraction V; Sigma) (optional)

3. Immediately prepare the following reaction mixture:

target DNA (200 μg/ml)	25 μl
H_2O	65 μl
10× DNAase/Mn^{++} digestion buffer	10 μl

Transfer 10 μl of the reaction mixture to a fresh microfuge tube containing 5 μl of 50 mM EDTA (pH 8.0). Store the tube on ice.

To the remainder of the reaction mixture, add 1.5 μl of diluted DNAase I (1 ng/μl) prepared in step 2. Incubate this reaction at 15°C.

4. After 1, 2, 5, 10, and 30 minutes of incubation, transfer 15 μl of the reaction to a fresh microfuge tube containing 5 μl of 50 mM EDTA (pH 8.0). Store the tubes on ice until all of the samples have been collected.

5. Analyze the size of the DNA fragments in each sample by electrophoresis of an aliquot (2 μl) through a 1.4% agarose gel. Use as marker pUC18 or pUC19 DNA that has been cleaved to completion with *Sau*3AI (see Appendix E).

6. Stain and photograph the gel, and pool the samples that contain a reasonable amount of fragments of the desired size (300 bp–1 kb).

7. Purify the DNA in the pooled sample by extraction with phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube, and add 5

volumes of H_2O (to reduce the concentration of Mn^{++} and EDTA). Add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. Add 2 volumes of ethanol at room temperature. Store the microfuge tube on ice for 10 minutes, and then collect the precipitate of DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Remove the supernatant, wash the pellet with 0.5 ml of 70% ethanol at room temperature, and recentrifuge. Remove as much as possible of the supernatant, and allow the last traces of ethanol to evaporate at room temperature. Redissolve the DNA in 25 μl of TE (pH 7.6).

8. Verify the size of the target DNA by analyzing an aliquot (2 μl) by electrophoresis through a 1.4% agarose gel. Use as marker pUC18 or pUC19 DNA that has been cleaved to completion with *Sau*3AI (see Appendix E).

9. Proceed with repair and size selection of DNA as described on page 13.30.

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1. To DNA (23 μl) that has been fragmented by sonication or by digestion with DNAase I in the presence of Mn^{++}, add:

a solution containing all four dNTPs, each at a concentration of 0.5 mM	3.0 μl
50 mM $MgCl_2$	3.0 μl
bacteriophage T4 DNA polymerase (10 units)	1.5 μl

Incubate the reaction for 15 minutes at room temperature, and then add 1 μl ($\sim$ 5 units) of the Klenow fragment of *E. coli* DNA polymerase I. Continue incubation for a further 15 minutes at room temperature.

The termini produced by sonication are extremely heterogeneous, consisting of both blunt-ended and frayed molecules with and without phosphate residues. Only a fraction of these molecules can be repaired by DNA polymerases, and the efficiency with which sonicated DNA can be cloned in bacteriophage M13 or phagemid vectors is therefore very low. Usually, however, 5–10 μg of sonicated, repaired, and size-selected target DNA yields several thousand recombinant clones. If insufficient clones are obtained, the problem is almost always due to the inability of DNA polymerases to repair the termini of the sonicated DNA. In this case, we recommend that a fresh batch of sonicated DNA be prepared and treated with the following enzymes in turn (1) calf intestinal alkaline phosphatase, (2) mung-bean nuclease, (3) bacteriophage T4 DNA polymerase in the presence of all four dNTPs, and finally (4) bacteriophage T4 polynucleotide kinase. The DNA should be purified by extraction with phenol and precipitation with ethanol after the addition of enzymes 1, 2, and 3.

2. Purify fragments of DNA of the desired size (300–600 bp) by electrophoresis through a 1.2% agarose gel. Use as marker pUC18 or pUC19 DNA that has been cleaved to completion with *Sau*3AI (see Appendix E).

To minimize the possibility of contamination, several lanes should be left empty between the fragmented target DNA and the marker and a fresh pipette tip should be used to load each lane of the gel.

3. Recover the target DNA from the gel by one of the elution methods described in Chapter 6. Redissolve the purified DNA in 10 μl of TE (pH 7.6). Check the integrity and recovery of the purified DNA by analyzing an aliquot (0.5 μl) by gel electrophoresis.

4. Ligate the target to the vector as described on page 13.33.

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Preparation of Vector DNA

1. In a total volume of 100 μl, digest 10 μg of vector DNA (phagemid or bacteriophage M13 replicative form DNA) with an enzyme that cleaves once within the polycloning site and generates blunt termini (e.g., digest M13mp18 and/or M13mp19 with *Sma*I). Analyze an aliquot of the digest in parallel with a sample of undigested DNA by electrophoresis to be sure that no circular forms of the DNA remain. Continue incubating the remainder of the digestion mixture at the appropriate temperature while the gel is running.

2. When digestion with the enzyme(s) is complete, add 10 μl of 100 mM Tris · Cl (pH 8.3), 10 mM $ZnCl_2$. Add 5 units of calf intestinal alkaline phosphatase (CIP), and incubate the reaction for 15 minutes at 37°C.

3. Add 2 μl of 0.5 M EDTA (pH 8.0) and 5 μl of 10% SDS. Add proteinase K to a final concentration of 50 μg/ml. Incubate for 30 minutes at 56°C.

   Proteinase K is used to digest the CIP, which must be completely removed if the subsequent ligation reactions are to work efficiently. An alternative method is to heat the reaction (at the end of step 2) to 65°C for 1 hour (or 75°C for 10 minutes) and then to extract once with phenol:chloroform.

4. Extract the reaction mixture once with phenol and once with phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube, and add 2 μl of 5 M NaCl. Add 2 volumes of ethanol at 0°C, and store the tube on ice for 10 minutes.

   NaCl is used instead of sodium acetate (pH 5.2) to prevent precipitation of EDTA.

5. Recover the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge. Remove the supernatant by aspiration, and add 1 ml of 70% ethanol at room temperature to the tube. Recentrifuge for a further 5 minutes. Carefully discard the supernatant, and store the open tube on the bench until the last traces of ethanol have evaporated. Redissolve the DNA in 200 μl of TE (pH 7.6). Check the recovery and integrity of the vector DNA by analyzing an aliquot (2 μl) by electrophoresis through a 0.8% agarose gel.

6. Carry out a series of test ligations to determine the effectiveness of the phosphatase treatment. Set up five reactions as follows:

Reaction	Type of DNA[a] A	B	C	H_2O (μl)	10× Ligation buffer (μl)	Bacteriophage T4 DNA ligase (μl)
1	+			6.5	1.0	0.5
2	+			7.0	1.0	
3		+		6.5	1.0	0.5
4		+		7.0	1.0	
5			+	5.0	1.0	0.5

[a]A = 100 ng of linearized, nonphosphatase-treated vector (in a volume of 2 μl); B = 100 ng of linearized, phosphatase-treated vector (in a volume of 2 μl); and C = 100 ng of bacteriophage λ DNA cleaved to completion with *Alu*I (in a volume of 2 μl).

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Incubate the ligation reactions for 12–16 hours at 16°C.

10 × Ligation buffer

500 mM Tris · Cl (pH 7.6)
100 mM $MgCl_2$
100 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

The efficiency of ligation can be increased by adding polyethylene glycol (PEG 8000; 30% w/v) to a concentration of 5% in the final ligation mixture (Pheiffer and Zimmerman 1983) (see Chapter 1, pages 1.70–1.71).

7. Transform (phagemid vectors) or transfect (bacteriophage M13 vectors; see Chapter 4 for test ligations) competent *E. coli* of the appropriate strain. Plate the transfected or transformed bacteria on media containing isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The following day, count the numbers of blue and white colonies (or colorless plaques).

Dephosphorylation should reduce the number of plaques or colonies by a factor of 50–100 (reaction 3 vs. reaction 1). Ligation of dephosphorylated vector to a standard preparation of blunt-ended fragments should result in a 10- to 50-fold increase in colorless plaques or white colonies.

8. If the results of the test ligations are satisfactory, dispense the dephosphorylated DNA into aliquots and store them at −20°C.

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Ligation to Vector DNA

1. Using the results of the test ligations (see pages 13.31–13.32) as a guide, set up a second series of test ligations containing 100 ng of dephosphorylated vector DNA and increasing concentrations of fragmented, blunt-ended target DNA.

2. Transform (phagemid vectors) or transfect (bacteriophage M13 vectors; see Chapter 4 for test ligations) competent *E. coli* of the appropriate strain. Plate the transfected or transformed bacteria on media containing IPTG or X-gal. The following day, count the numbers of blue and white colonies (or colorless plaques).

   The number of recombinants obtained with fragmented, blunt-ended target DNA will be approximately tenfold lower than the number obtained with a standard preparation of blunt-ended DNA.

   The aim of this test ligation is to find a concentration of fragmented target DNA that minimizes the number of recombinants containing artificially fused target fragments, which can greatly complicate random sequencing. It is therefore essential not to use saturating quantities of target DNA. Instead, determine the amount of target DNA that will produce a modest increase (approximately fivefold) in the number of recombinant clones over background.

3. When the results of the second series of test ligations are available, set up a large-scale ligation that will yield sufficient recombinant clones to complete the sequencing project.

4. Transform (phagemid vectors) or transfect (bacteriophage M13 vectors) competent *E. coli* of the appropriate strain. Plate the transfected or transformed bacteria on media containing IPTG and X-gal. The following day, count the numbers of blue and white colonies (or colorless plaques).

   Bacterial colonies carrying phagemids can be stored at 4°C for a few weeks on the original agar plates. However, recombinant bacteriophage M13 plaques should be picked as soon as possible, since the bacteriophage particles can diffuse considerable distances through top agar. Plaques grown for extended periods of time (> 12–16 hours) at 37°C or stored for a few days at 4°C frequently become contaminated. There is an increase in both the intensity and number of background bands when single-stranded DNAs prepared from old plaques are used in sequencing reactions. To minimize the possibility of cross contamination, it is important to pick plaques that are well-separated from their neighbors.

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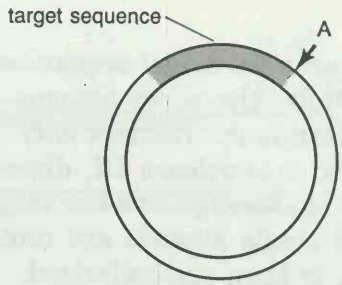
GENERATION OF NESTED SETS OF DELETION MUTANTS

Several methods have been developed to generate nested sets of deletion mutants that lack progressively more nucleotides from one end or the other of the target DNA. These methods all rely on the following nucleases that digest DNA in a predictable fashion:

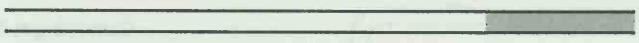
- *BAL 31.* This enzyme carries both an exonucleolytic activity that progressively degrades double-stranded linear DNA by liberating mononucleotides from the 3′ termini and a weaker single-strand-specific endonucleolytic activity. Thus, the products of digestion of double-stranded DNA with BAL 31 are truncated molecules carrying blunt ends or short, protruding 5′ termini. To generate deletion mutants, a recombinant plasmid (or bacteriophage M13 replicative form DNA) is linearized with a restriction enzyme that cleaves at one end of the target sequence. The double-stranded linear DNA is digested with BAL 31 for different lengths of time and the truncated fragments of target DNA are then cleaved from the remainder of the vector with a second restriction enzyme and recloned (see Figure 13.5).

- *Pancreatic DNAase I.* In the presence of Mg^{++}, DNAase I behaves like an endonuclease that introduces nicks independently into each strand of double-stranded DNA. However, in the presence of a transition metal ion, such as Mn^{++} or Co^{++}, the enzyme cuts both strands of DNA at approximately the same place (Melgar and Goldthwait 1968). This property can be used to create deletions in a segment of target DNA that has been cloned in a plasmid or bacteriophage M13 vector (Frischauf et al. 1980; Hong 1982, 1987; Labeit et al. 1987). Because closed circular DNA is cleaved more efficiently than linear molecules and because the sites of cleavage are distributed in a statistically random fashion, the population of closed circular DNAs is converted by DNAase I into a permuted set of linear molecules. These are then digested with a restriction enzyme whose unique site of cleavage lies at one end of the target DNA. Recircularization of the resulting population generates clones that lack sequences lying between the site of DNAase I cleavage and the restriction site (see Figure 13.6).

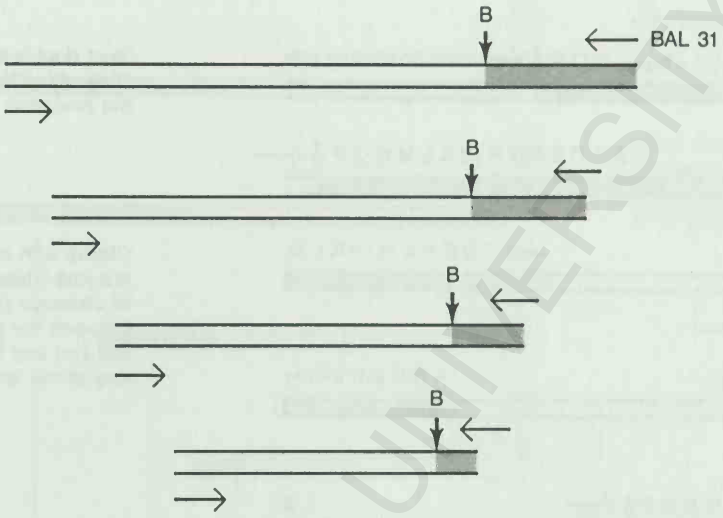
- *Exonuclease III.* This enzyme catalyzes the stepwise removal of 5′ mononucleotides from the recessed or blunt 3′-hydroxyl termini of double-stranded DNA (Weiss 1976). However, protruding 3′ termini are completely resistant to the activity of the enzyme (Rogers and Weiss 1980). This property of exonuclease III has been used to develop a protocol that allows construction of unidirectional deletions without subcloning the target DNA. To create unidirectional deletion mutants (Henikoff 1984, 1987), the double-stranded DNA of a recombinant plasmid or bacteriophage M13 replicative form DNA is digested with two restriction enzymes whose sites of cleavage both lie between one end of the target DNA and the primer-binding site. The enzyme that cleaves nearer the target sequences must generate a blunt



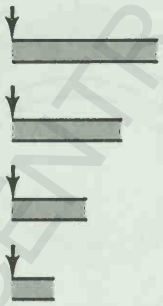
Digest plasmid DNA with a restriction enzyme (A) that cleaves at one end of the target sequence.



Digest linear DNA with BAL 31 for different lengths of time



Repair termini of target fragments with DNA polymerases.
Recover truncated target sequence by digestion with a restriction enzyme (B) that cleaves at the opposite end of the target sequence.



Clone fragments in a plasmid, phagemid, or bacteriophage M13 vector.

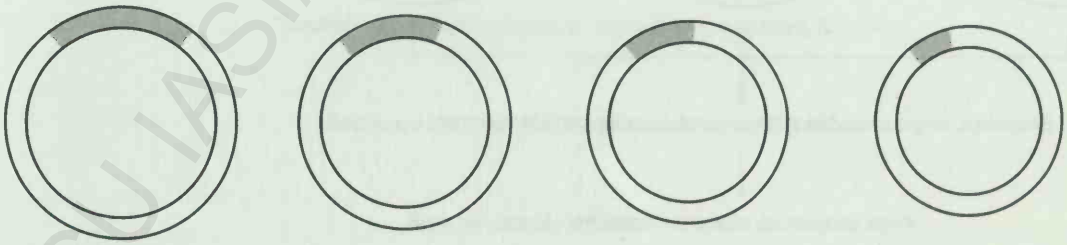


FIGURE 13.5
Generation of nested deletion mutants with BAL 31.

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The enzyme that cleaves nearer the target sequences must generate a blunt end or a recessed 3′ terminus; the other enzyme must generate a 3- or 4-nucleotide protruding 3′ terminus. Because only one end of the resulting linear DNA is susceptible to exonuclease III, digestion proceeds unidirectionally away from the site of cleavage into the target DNA sequences (see Figure 13.7). The exposed single strands are removed by digestion with nuclease S1, and the DNA is then recircularized. If desired, a synthetic linker can be inserted at the site of recircularization.

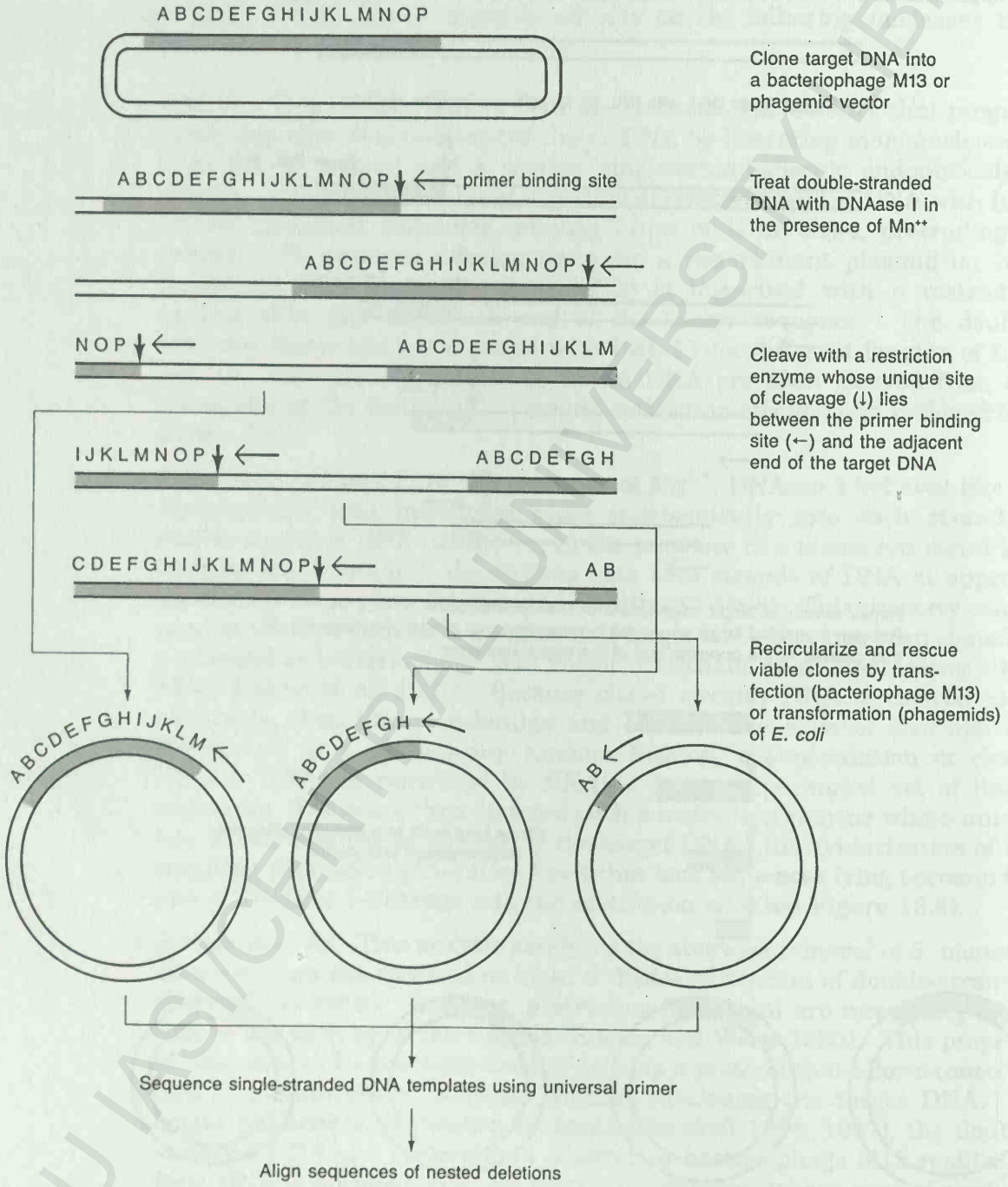
ABCDEFGHIJKLMNOP
Clone target DNA into a bacteriophage M13 or phagemid vector
ABCDEFGHIJKLMNOP
primer binding site
Treat double-stranded DNA with DNAase I in the presence of Mn++
ABCDEFGHIJKLMNOP
NOP
ABCDEFGHIJKLM
Cleave with a restriction enzyme whose unique site of cleavage (↓) lies between the primer binding site (←) and the adjacent end of the target DNA
IJKLMNOP
ABCDEFGH
CDEFGHIJKLMNOP
AB
Recircularize and rescue viable clones by transfection (bacteriophage M13) or transformation (phagemids) of E. coli
ABCDEFGHIJKLM
ABCDEFGH
AB
Sequence single-stranded DNA templates using universal primer
Align sequences of nested deletions

FIGURE 13.6
Generation of nested deletion mutants with DNAase I in the presence of Mn^{++}.

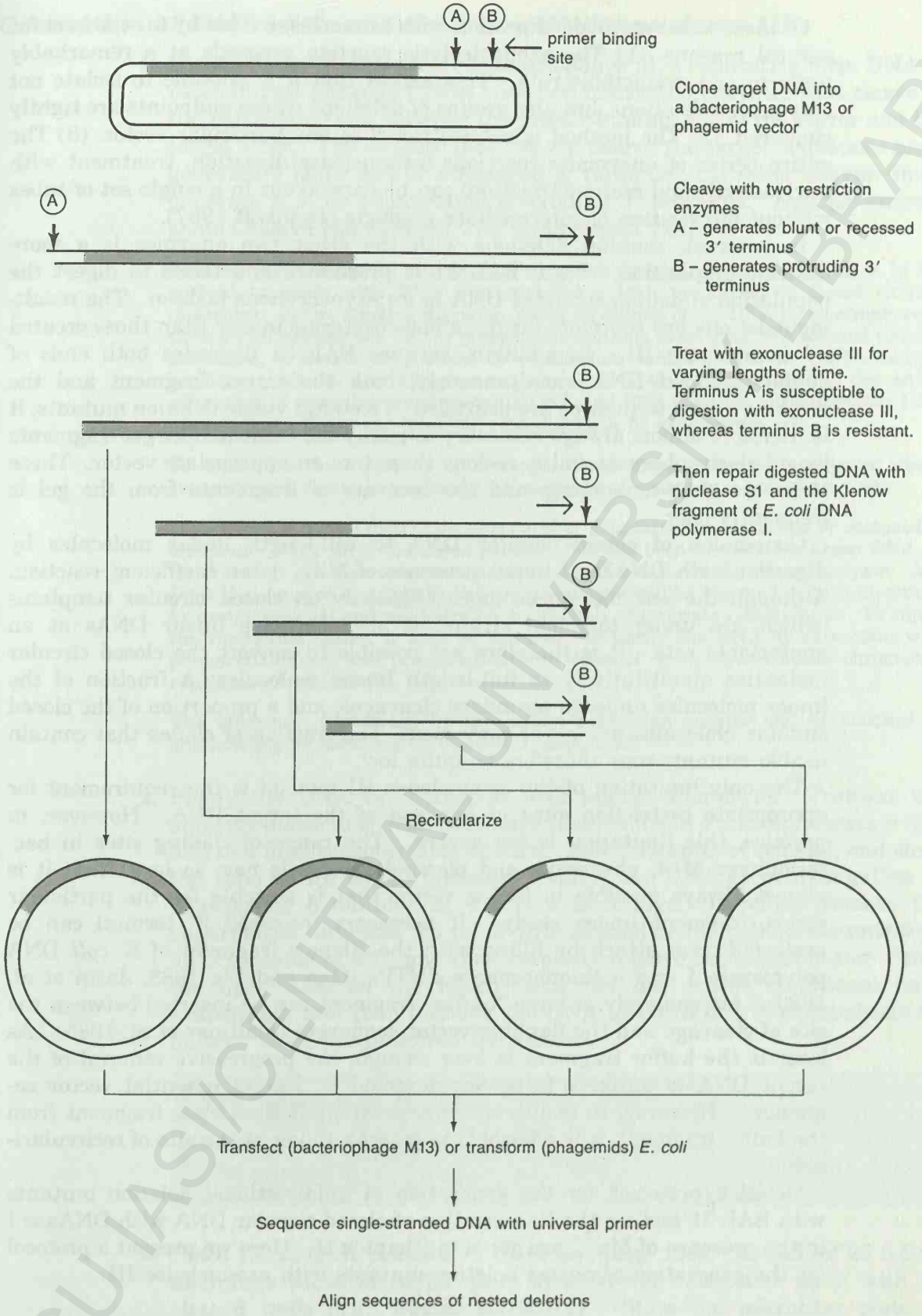
A
B
primer binding site
Clone target DNA into a bacteriophage M13 or phagemid vector
A
B
Cleave with two restriction enzymes
A – generates blunt or recessed 3′ terminus
B – generates protruding 3′ terminus
B
Treat with exonuclease III for varying lengths of time. Terminus A is susceptible to digestion with exonuclease III, whereas terminus B is resistant.
B
Then repair digested DNA with nuclease S1 and the Klenow fragment of E. coli DNA polymerase I.
B
B
B
Recircularize
Transfect (bacteriophage M13) or transform (phagemids) E. coli
Sequence single-stranded DNA with universal primer
Align sequences of nested deletions

FIGURE 13.7
Generation of nested deletion mutants with exonuclease III.

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Of these three methods, digestion with exonuclease III is by far the best for several reasons. (1) The exonucleolytic reaction proceeds at a remarkably uniform and predictable rate. This means that it is possible to isolate not only nested deletions, but also groups of deletions whose endpoints are tightly clustered. (2) The method is not restricted to any particular vector. (3) The entire series of enzymatic reactions (exonuclease digestion, treatment with nuclease S1, and recircularization) can be carried out in a single set of tubes without purification of intermediate products (Henikoff 1987).

In contrast, making deletions with the other two enzymes is a more difficult proposition. First, BAL 31 is processive and tends to digest the population of double-stranded DNA in an asynchronous fashion. The resulting deletions are therefore far more heterogeneous in size than those created by exonuclease III. In addition, because BAL 31 degrades both ends of double-stranded DNA simultaneously, both the target fragment and the flanking vector sequences are degraded. To obtain viable deletion mutants, it is therefore almost always necessary to purify the truncated target fragments by gel electrophoresis and to reclone them into an appropriate vector. These steps are time-consuming, and the recovery of fragments from the gel is frequently poor.

Conversion of closed circular DNA to full-length linear molecules by digestion with DNAase I in the presence of Mn^{++} is an inefficient reaction. Although the enzyme works more efficiently on closed circular templates (which are under torsional strain), it also degrades linear DNAs at an appreciable rate. It is therefore not possible to convert the closed circular molecules quantitatively to full-length linear molecules: A fraction of the linear molecules undergo secondary cleavages, and a proportion of the closed circular molecules are never converted. The fraction of clones that contain usable mutants may therefore be quite low.

The only limitation of the exonuclease III method is the requirement for appropriate restriction sites at one end of the target DNA. However, in practice, this limitation is not severe. The range of cloning sites in bacteriophage M13, phagemid, and plasmid vectors is now so large that it is almost always possible to find a vector that is suitable for the particular target fragment under study. If necessary, recessed 3′ termini can be protected from attack by filling with the Klenow fragment of *E. coli* DNA polymerase I and α-thiophosphate dNTPs (Guo and Wu 1983; Jasin et al. 1983). Alternatively, a large "buffer" fragment can be inserted between the site of cleavage and the flanking vector sequences (Haltiner et al. 1985). As long as the buffer fragment is long enough, the progressive removal of the target DNA is unlikely to be accompanied by loss of essential vector sequences. However, to facilitate the separation of the target fragment from the buffer fragment, it is advisable to insert a linker at the site of recircularization.

Detailed protocols for the generation of unidirectional deletion mutants with BAL 31 and for the linearization of closed circular DNA with DNAase I in the presence of Mn^{++} are given in Chapter 15. Here we present a protocol for the generation of nested deletion mutants with exonuclease III.

Generation of Nested Sets of Deletions with Exonuclease III

1. Digest 10 μg of recombinant bacteriophage M13 replicative form DNA or recombinant phagemid DNA with two restriction enzymes that cleave in the polycloning site between the primer-binding site of the vector and the target DNA. The enzyme that cleaves nearer the target sequences must generate a blunt end or a recessed 3′ terminus; the other enzyme must generate a 3- or 4-nucleotide protruding 3′ terminus. Neither enzyme should cleave elsewhere in the recombinant DNA.

   Because exonuclease III will initiate digestion from single-strand nicks, it is important to begin with DNA that contains a high proportion of closed circular molecules. Before starting digestion, check an aliquot of the DNA by electrophoresis through an agarose gel. If the preparation contains more than 10% relaxed circular molecules, it should be purified by equilibrium centrifugation in CsCl–ethidium bromide gradients (see Chapter 1, pages 1.42–1.45). This step has the added advantage of removing small pieces of DNA and RNA from the closed circular DNA preparation. These can interfere with digestion by exonuclease III.

   To maximize the efficiency of cleavage, avoid using restriction enzymes whose recognition sites are immediately adjacent to each other in a polycloning site.

   Digest first with the restriction enzyme that generates the blunt end or recessed 3′ terminus. When all of the closed circular DNA has been converted to linear DNA, as confirmed by gel electrophoresis, adjust the buffer and add the second enzyme. Any DNA that escapes cleavage by the second enzyme will be digested in both directions by exonuclease III and will therefore be unlikely to generate viable clones. To adjust the restriction buffer, it may be necessary to purify the DNA by extraction with phenol:chloroform and precipitation with ethanol or by spun-column chromatography (see Appendix E).

   The efficiency of cleavage by the second restriction enzyme can be checked by end-labeling as discussed in Chapter 1, page 1.56.

2. Purify the DNA by extraction with phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube, and add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M. Add 2 volumes of ethanol, and store the tube on ice for 10 minutes. Collect the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge. Carefully remove the supernatant, and add 0.5 ml of 70% ethanol to the pellet. Recentrifuge for 2 minutes at 4°C, and then carefully remove the supernatant. Store the open tube on the bench until the last traces of ethanol have evaporated, and then dissolve the DNA in 60 μl of 1 × exonuclease III buffer. Store the dissolved DNA on ice.

   10 × Exonuclease III buffer

   0.66 M Tris · Cl (pH 8.0)
   66 mM $MgCl_2$

3. Place 7.5 μl of S1 reaction mixture (see page 13.40) in each of 25 0.5-ml microfuge tubes or in 25 wells in a 96-well microtiter plate with U-shaped wells (e.g., Baxter B1190-17). Store the microtiter plate or microfuge tubes on a bed of ice.

S1 reaction mixture contains:

H_2O	172 μl
10× S1 buffer	27 μl
nuclease S1	60 units

10× S1 buffer

5 M NaCl	5.0 ml
3 M potassium acetate (pH 4.5)	1.1 ml
glycerol	5.0 ml
1 M $ZnSO_4$	20.0 μl

4. Incubate the DNA solution prepared in step 2 for 5 minutes at 37°C. Transfer 2.5 μl of the solution to the first microfuge tube or well of the microtiter plate containing the S1 reaction mixture. To the remainder of the DNA solution, add 150 units of exonuclease III per picomole of recessed 3′ termini. Vortex briefly to mix the contents of the tube, and immediately return the tube to the 37°C water bath.

5. At 30-second intervals, remove 2.5-μl samples of the DNA solution and place in successive microfuge tubes or wells of the microtiter plate containing the S1 reaction mixture.

   Under these conditions, the amount of exonuclease III is saturating, and approximately 200 nucleotides per minute are removed from the blunt end or recessed 3′ terminus of each of the DNA molecules in the solution. More or less DNA can be removed by varying the intervals between successive samples.

6. After all of the samples have been taken, incubate the microfuge tubes or microtiter plate for 30 minutes at 30°C. Add 1 μl of S1 stop mixture to each of the microfuge tubes or wells, and incubate for 10 minutes at 70°C. This inactivates nuclease S1 and any residual exonuclease III.

S1 stop mixture

0.3 M Tris base
50 mM EDTA (pH 8.0)

7. Transfer the microfuge tubes or microtiter plate to a bed of ice. Analyze aliquots of each of the samples by agarose gel electrophoresis. Choose a concentration of agarose that will allow maximum discrimination between the original DNA and fragments that are smaller in size by up to 2 kb.

8. Pool the samples containing DNA fragments of the desired size. Add 1 μl of Klenow mixture for each 10 μl of pooled sample. Incubate the pooled sample for 5 minutes at 37°C.

   Klenow mixture (sufficient for 30 samples) contains:

H_2O	20 μl
1 M $MgCl_2$	6 μl
0.1 M Tris · Cl (pH 7.6)	3 μl
Klenow fragment of *E. coli* DNA polymerase I	3 units

During this brief incubation in the absence of dNTPs, the 3′→5′ exonuclease activity of the Klenow fragment of *E. coli* DNA polymerase I removes any remaining protruding 3′ termini from the digested DNA.

9. For each 10 μl of pooled sample, add 1 μl of a solution containing the four dNTPs, each at a concentration of 0.5 mM. Continue incubation for a further 15 minutes at room temperature.

10. Add 40 μl of ligase mixture for each 10 μl of pooled sample. Mix, and continue incubation at room temperature for a further 2 hours.

Ligase mixture (sufficient for 24 samples) contains:

H_2O	550 μl
10× ligation buffer	100 μl
5 mM rATP	100 μl
30% w/v polyethylene glycol (PEG 8000)	250 μl
bacteriophage T4 DNA ligase	5 Weiss units

10× Ligation buffer

500 mM Tris · Cl (pH 7.6)
100 mM $MgCl_2$
100 mM dithiothreitol
500 μg/ml bovine serum albumin (Fraction V; Sigma) (optional)

This buffer should be stored in small aliquots at −20°C.

For definition of Weiss unit, see page 13.25.

11. Transform (phagemid vectors) or transfect (bacteriophage M13 vectors) competent *E. coli* of the appropriate strain (see Chapter 4). Using 5–10 μl of the pooled sample, prepare minipreparations of bacteriophage M13 replicative form DNA or phagemid DNA from at least 18 randomly selected plaques or colonies (see Chapter 1, pages 1.25–1.31). Linearize the DNAs by digestion with an appropriate restriction enzyme and analyze their sizes by electrophoresis on a 1% agarose gel. Use as marker the original plasmid DNA that has been linearized by restriction enzyme digestion. Choose clones of an appropriate size for sequencing.

Approximately 80–90% of the deletions retain the primer-binding site and can be sequenced using a universal primer.

It is best to select clones for sequencing that differ in size by approximately 200 bp. Such clones are likely to form an overlapping set.

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Sequencing by the Sanger Dideoxy-mediated Chain-termination Method

SETTING UP DIDEOXY-MEDIATED SEQUENCING REACTIONS

Preparation of Single-stranded DNA

Small-scale preparations of single-stranded bacteriophage M13 or phagemid DNAs should be isolated from bacteriophage particles synthesized by individual plaques or from bacterial colonies as described in Chapter 4. The yields of DNA are normally sufficient for 5–10 sets of sequencing reactions. Before setting up sequencing reactions, it is advisable to check the amount of single-stranded template in each preparation of single-stranded bacteriophage M13 or phagemid DNA. This can be done by analyzing an aliquot of each preparation of DNA by electrophoresis through a 1% agarose gel, using as marker a standard preparation of bacteriophage M13 DNA. Although single-stranded bacteriophage M13 DNA runs on an agarose gel as a broad fuzzy band that stains poorly with ethidium bromide, it is nevertheless quite easy to estimate the relative quantities of DNA in different preparations.

Preparation of Primers

Many companies supply universal primers as lyophilized powders, which should be dissolved at a concentration of 2 μg/ml in 10 mM Tris · Cl (pH 7.4), 5 mM NaCl, 0.1 mM EDTA (pH 8.0). Some companies supply universal primers already in solution. Solutions of primers should be stored at −20°C. (*Note*: 1 μg of universal primer [19-mer] is approximately equal to 160 pmoles.)

Primers of several different lengths (from 15 to 26 nucleotides) are available from a number of different commercial suppliers. There is no detectable difference in the quality of the sequence obtained from recombinant bacteriophage M13 templates with primers of different lengths. However, when denatured double-stranded DNA templates such as bacteriophage λ or plasmids are used, longer primers (from 25 to 29 nucleotides) give rise to fewer artifactual bands.

Primers synthesized in the laboratory generally need not be purified by gel electrophoresis before use. The amount of contamination by "off-size" oligonucleotides is usually too small ($< 10\%$) to affect the quality of the DNA sequence. However, it is often necessary to experiment with laboratory-synthesized primers to determine the optimum ratio of primer to template in the sequencing reaction. Laboratory-synthesized primers should be dissolved at a concentration of 2 μg/ml as described above and stored at −20°C.

Microtiter Plates

Dideoxy-mediated sequencing reactions using the Klenow fragment of *E. coli* DNA polymerase I or reverse transcriptase are carried out in two stages: an *annealing reaction*, in which the oligonucleotide primer is hybridized to the

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template DNA, and four *chain-extension/chain-termination reactions*, in which the annealed oligonucleotide is extended and the newly synthesized chains of DNA are terminated by incorporation of ddATP, ddGTP, ddCTP, or ddTTP. In sequencing reactions using Sequenases, a preliminary step—an *incorporation reaction*—is included to ensure efficient incorporation of radioactive precursor in the newly synthesized DNA.

Until a few years ago, chain-extension/chain-termination reactions were carried out in conventional microfuge tubes—a procedure that works well when only one or two sets of reactions are being processed but is extremely tedious and inefficient when larger numbers of templates are sequenced. This problem can be solved by carrying out the chain-extension/chain-termination reactions in disposable microtiter plates with 96 U-shaped wells (e.g., Baxter B1190-17). The reagents are deposited as separate droplets on the side of each well, toward the rim, and the reactions are initiated simultaneously by briefly centrifuging the plates in a low-speed centrifuge equipped with microtiter plate buckets. This sediments the droplets on the bottom of the well and mixes the components of the reaction. Microtiter plates have the following advantages: (1) Up to 24 sets of chain-extension/chain-termination reactions can be processed simultaneously, (2) reagents can be added using repeating dispenser pipettes, and (3) many sets of sequencing reactions can be carried out synchronously.

Some laboratories carry out all stages of sequencing reactions—from annealing to chain termination—in microtiter plates. In our experience, this is not advisable. During annealing, when small volumes of solutions are heated to 55°C, evaporation from the wells of a microtiter plate can be a problem. We therefore recommend that annealing of primer to template be carried out in closed microfuge tubes and that the subsequent enzymatic reaction be set up in microtiter wells.

Chain-extension/Chain-termination Reaction Mixtures

The competition between chain elongation and termination is determined by the ratio of dNTP to ddNTP in each of the four sequencing reactions. Each of the DNA polymerases used for dideoxy-mediated sequencing has a different affinity for both conventional dNTPs and chain-terminating ddNTPs. Thus, reaction mixtures that give good results with one enzyme are unlikely to work well with another. It is almost always necessary to "fine-tune" the concentration of ddNTPs to obtain optimal results for the particular task at hand. When sequencing for the first time with a particular enzyme (either Sequenases or the Klenow fragment of *E. coli* DNA polymerase I), we recommend purchasing a kit from a commercial manufacturer that contains the relevant DNA polymerase, pretested chain-extension/chain-termination mixtures, single-stranded templates, and oligonucleotide primers. As components of the kit are used up, we recommend that they be replaced with homemade materials, which are less expensive and can be made in a wide range of concentrations. Each new batch of reagents should be checked by setting up a series of test reactions with a standard template and primer and a range of concentrations of dNTPs and ddNTPs. Initially, it is best to use fixed concentrations of dNTPs and variable concentrations of ddNTPs. Often, several series of test reactions are necessary to define optimal conditions.

STOCK SOLUTIONS OF dNTPs AND ddNTPs

The following stock solutions are used to prepare the working solutions of dNTPs and ddNTPs (see page 13.60) for sequencing reactions using either the Klenow fragment of *E. coli* DNA polymerase I or Sequenase.

Stock solutions of dNTPs contain a single dNTP (sodium salt) at a concentration of 0.5 mM in TE (pH 8.0):

0.5 mM dATP	302 μg/ml
0.5 mM dCTP	284 μg/ml
0.5 mM dGTP	304 μg/ml
or	
0.5 mM dITP	288 μg/ml
0.5 mM dTTP	291 μg/ml

Stock solutions of ddNTPs contain a single ddNTP at a concentration of 10 mM in TE (pH 8.0):

10 mM ddATP	5.7 mg/ml
10 mM ddCTP	5.4 mg/ml
10 mM ddGTP	5.7 mg/ml
10 mM ddTTP	5.5 mg/ml

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DENATURING POLYACRYLAMIDE GELS

Both the amount and the accuracy of the nucleotide sequence are determined by the quality of the polyacrylamide gels used to display the radiolabeled DNA fragments generated by chemical and enzymatic sequencing reactions. Under optimal conditions, between 300 and 400 nucleotides of reliable sequence can be obtained from a denaturing polyacrylamide gel. However, by electrophoresing aliquots of the sequencing reaction for different lengths of time, it is possible to read up to 500 nucleotides of sequence from a single gel. This high degree of resolution can be obtained reproducibly only if care is taken in the preparation and handling of the gels.

The concentration of acrylamide used to prepare the gel depends on the size of the DNA fragments that are to be analyzed. To read sequence within 50 nucleotides of the 5′ terminus of the primer (or within 50 nucleotides of the radiolabeled terminus in the case of Maxam-Gilbert sequencing), gels should be cast with high (12–20%) concentrations of acrylamide. Sequences lying between 25 and 400 nucleotides from the terminus of the primer can be read from gels containing 6% acrylamide. More distant sequences can be determined from gels cast with 4% or 5% polyacrylamide.

Sequencing gel apparatuses come in many sizes and shapes to suit individual tastes. The major variables are:

- *The length of the gel.* A length of 40–50 cm is standard, but apparatuses that accommodate gels up to 100 cm in length are available. However, long gels are extremely difficult to handle and require special, large containers for fixation and exposure to X-ray film.
- *The width of the gel.* Narrow gels (20 cm) can contain up to 40 lanes and are easier to handle, but wide gels (40 cm) will accept twice as many samples.
- *The thickness of the gel.* The thickness of sequencing gels is defined by the thickness of the thin plastic strips that are used as spacers between the front and back glass plates. Standard sequencing gels are 0.3–0.4 mm thick. Thinner gels (0.2 mm) give excellent resolution, but they are fragile; thicker gels (0.6 mm) will accept larger volumes of samples, but they are more difficult to fix and dry.
- *The cross-sectional shape of the gel.* Most sequencing gels are of constant thickness. On such gels, the logarithmic relationship between the length of a fragment of DNA and its mobility produces widely spaced bands at the bottom of the gel and crowded bands at the top. This limits the amount of reliable nucleotide sequence that can be read from a single gel. One way to alleviate this problem is to use tapered or wedge-shaped gels that are thicker at the bottom (0.6–0.75 mm) than at the top (0.25 mm) (Bankier and Barrell 1983; Reed et al. 1986). Wedge-shaped gels compress the spacing between adjacent bands of DNA by lowering the electrical resistance, and thus the voltage drop, per unit length of the gel. The DNA therefore slows down as the thickness of the gel increases. Despite their increased resolution, wedge-shaped gels are not used routinely for sequencing in most laboratories. Wedge-shaped gels take much longer to dry than normal gels, and they often crack on the drying apparatus. Furthermore,

the resolution of bands at the bottom of the gel is sometimes poor. Fortunately, there is another way to reduce the spacing between the bands of DNA at the bottom of the gel, i.e., by progressively increasing the concentration of buffer in the lower part of the gel (Biggin et al. 1983; Hong 1987). Because the voltage carried through the gel decreases with increasing ionic strength, DNA moves more slowly as it approaches the bottom of the gel. This decreases the spacing between adjacent bands of DNA and increases the total number of nucleotides that can be read from a single gel by approximately 30%.

- *The loading slots.* Two types of slots can be used in sequencing gels:

  1. Conventional wells that are formed by inserting a plastic template into the top of the acrylamide solution before it polymerizes. After the template is removed from the gel, the slots are washed with buffer to remove any unpolymerized acrylamide and the samples are then loaded. The samples are therefore separated from each other by fingers of polyacrylamide.

  2. Slots that are created by inserting a shark's tooth comb into the top of a gel after it has polymerized. The points of the teeth serve as barriers to prevent the mixing of adjacent samples of DNA. This design reduces the risk of torn or deformed wells and generates a flatter, more uniform surface for loading. Furthermore, the shark's tooth comb leaves virtually no separation between adjacent lanes, facilitating accurate reading of the sequence. The disadvantages are that loading of samples is more difficult and leakage is sometimes a problem. The first of these difficulties can be overcome with practice. The second problem arises when the spacers used to form the gel are slightly thicker than the shark's tooth comb. Combs and spacers should therefore be kept as matched sets.

- *The temperature gradient across the gel.* During electrophoresis, a temperature gradient is established as the gel heats up. Unless the apparatus has a mechanism to diffuse the heat evenly, the center of the gel becomes hotter than the edges, resulting in curved bands of DNA. The simplest temperature-control systems are plastic-coated metal plates that are clamped to the glass plates holding the gel. In more elaborate apparatuses, the electrophoresis buffer is used to cool the back of the gel, which can then be run at a predetermined temperature.

Preparation of Buffer-gradient Polyacrylamide Gels

The method given below describes how to pour and run sequencing gels that contain increasing concentrations of buffer toward the base of the gel (Biggin et al. 1983; Hong 1987). It can easily be adapted to all commonly used makes of apparatuses. Sequencing gels should be made at least 2 hours before use and can be made up to 24 hours before they are needed.

1. Prepare the following stock solutions:

40% Acrylamide solution

acrylamide (DNA-sequencing grade)	380 g
N,N'-methylenebisacrylamide	20 g
distilled H_2O to 600 ml	

Heat the solution to 37°C to dissolve the chemicals. Adjust the volume to 1 liter with distilled water. Filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size), and store in dark bottles at room temperature.

Caution: Acrylamide is a potent neurotoxin and is absorbed through the skin. The effects of acrylamide are cumulative. Wear gloves and a mask when weighing powdered acrylamide and methylenebisacrylamide. Wear gloves when handling solutions containing these chemicals. Although polyacrylamide is considered to be nontoxic, it should be handled with care because of the possibility that it might contain small quantities of unpolymerized acrylamide.

Cheaper grades of acrylamide and bisacrylamide are often contaminated with metal ions. Stock solutions of acrylamide can easily be purified by stirring overnight with about 0.2 volume of monobed resin (MB-1, Mallinckrodt), followed by filtration though Whatman No. 1 paper.

During storage, acrylamide and bisacrylamide are slowly converted to acrylic acid and bisacrylic acid. This deamination reaction is catalyzed by light and alkali. Check that the pH of the acrylamide solution is 7.0 or less, and store the solution in dark bottles at room temperature. Fresh solutions should be prepared every few months.

5 × TBE

Tris base	54 g
boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
deionized H_2O to 1 liter	

TBE is used at a working strength of 1 × (89 mM Tris-borate, 2 mM EDTA) for polyacrylamide gel electrophoresis. This is twice the strength usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for polyacrylamide gel electrophoresis are fairly small, and the amount of electric current passed through them is often considerable. 1 × TBE is required to provide adequate buffering power. The pH of the buffer should be ~8.3.

The same stock of 5 × TBE should be used to prepare both the gel and the running

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buffers. Small differences in ionic strength or pH produce buffer fronts that can greatly distort the DNA bands.

10% Ammonium persulfate

Ammonium persulfate	1 g
H_2O to	10 ml

The solution may be stored at 4°C for several weeks.

6% Acrylamide/urea top solution

40% acrylamide solution	75 ml
5× TBE	50 ml
urea (ultrapure)	230 g

Adjust the volume to 500 ml with deionized water, and filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size). The 6% acrylamide/urea top solution may be stored for several weeks at 4°C.

6% Acrylamide/urea bottom solution

40% acrylamide solution	30 ml
5× TBE	100 ml
urea (ultrapure)	92 g
sucrose	20 g
bromophenol blue	10 mg

Filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size). The 6% acrylamide/urea bottom solution may be stored for several weeks at 4°C.

The sucrose is added to increase the density of the bottom solution and to facilitate pouring of the buffer gradient. The bromophenol blue serves as a visible marker to check that the buffer gradient has formed correctly.

2. Prepare the number of glass plates required to accommodate the sequencing reactions. If it is necessary to remove old silicon from the plates, swab them with KOH/methanol, which is prepared by adding ~5 g of KOH pellets to 100 ml of methanol.

Caution: Handle the KOH and the KOH/methanol solutions with great care. Use gloves and a face protector.

Then wash the glass plates and spacers in warm detergent solution, and rinse them thoroughly in tap water, followed by deionized water. Hold the plates by their edges so that material from the gloves or oils from your hands do not become deposited on the working surfaces of the plates. Rinse the plates with ethanol and set them aside to dry. The plates must be cleaned meticulously to ensure that air bubbles do not form when the gel is poured.

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Treat one surface of each plate with silicone solution. This prevents the gel from sticking tightly to both plates and reduces the possibility that the gel will tear when it is removed from the mold when electrophoresis is completed.

To siliconize a plate, lay the plate on a pad of paper in a chemical hood, and pour a small quantity of siliconizing fluid (e.g., Sigmacote) onto it. Wipe the fluid over the surface of the plate with a pad of Kimwipes, and then rinse the plate in deionized water. Dry the plate with a hairdryer. Wear gloves during siliconization.

3. There are many types of electrophoresis apparatuses available commercially, and the arrangement of the glass plates and spacers differs slightly from manufacturer to manufacturer. In all cases, the aim is to form a watertight seal between the plates and spacers, so that the unpolymerized gel solution does not leak out. Typically, the two plates are of slightly different size and one of them is notched. Spacers are made of thin (usually 0.4-mm) flexible plastic.

   Lay the larger (or unnotched) plate flat on the bench and arrange the two spacers in place along the sides (see Figure 13.8). A couple of *minute* dabs of petroleum jelly help to keep the spacers in position during the next steps. Make sure there is no dust on the plates, and then lay the smaller (or notched) plate in position, resting on the spacers. Clamp together one side of the plates with several large (5-cm length) bulldog binder clips. Bind the entire length of the other side and the bottom of the plates with gel-sealing tape (3M yellow electrical tape; BRL) to make a watertight seal. Take particular care with the bottom corners of the plates, since these are the places where leaks most often occur. Transfer the bulldog clips to the sealed side of the plates. Bind the remaining side of the plates with gel-sealing tape.

   Several other methods have been used to prevent leakage of acrylamide solution from assembled gel molds, including:
   - Sealing the edges with agarose.
   - Inserting a plastic spacer into the open space at the bottom of the mold, sealing with tape, and then clamping the plates together with bulldog clips. (*Note:* A bulldog clip should be used on the bottom of the gel mold *only* when this third spacer is in place; otherwise, the glass plates may crack.)
   - Sealing the bottom of the plate with a strip of filter paper impregnated with catalyzed acrylamide (Wahls and Kingzette 1988).

   We recommend that you use whichever of these methods you find to be most reliable with the particular type of gel mold that is available.

4. Place the comb into the open end of the gel mold and test to see that it fits snugly. Remove the comb and lay the empty gel mold on the bench.

5. Cover the working area with plastic-backed protective paper. It is almost impossible to pour sequencing gels without dripping acrylamide solutions onto the bench. Prepare a sequencing gel as described on pages 13.52–13.53. The preparation of the gel must be done without interruption.

   Caution: Wear gloves when preparing and handling the gel solutions.

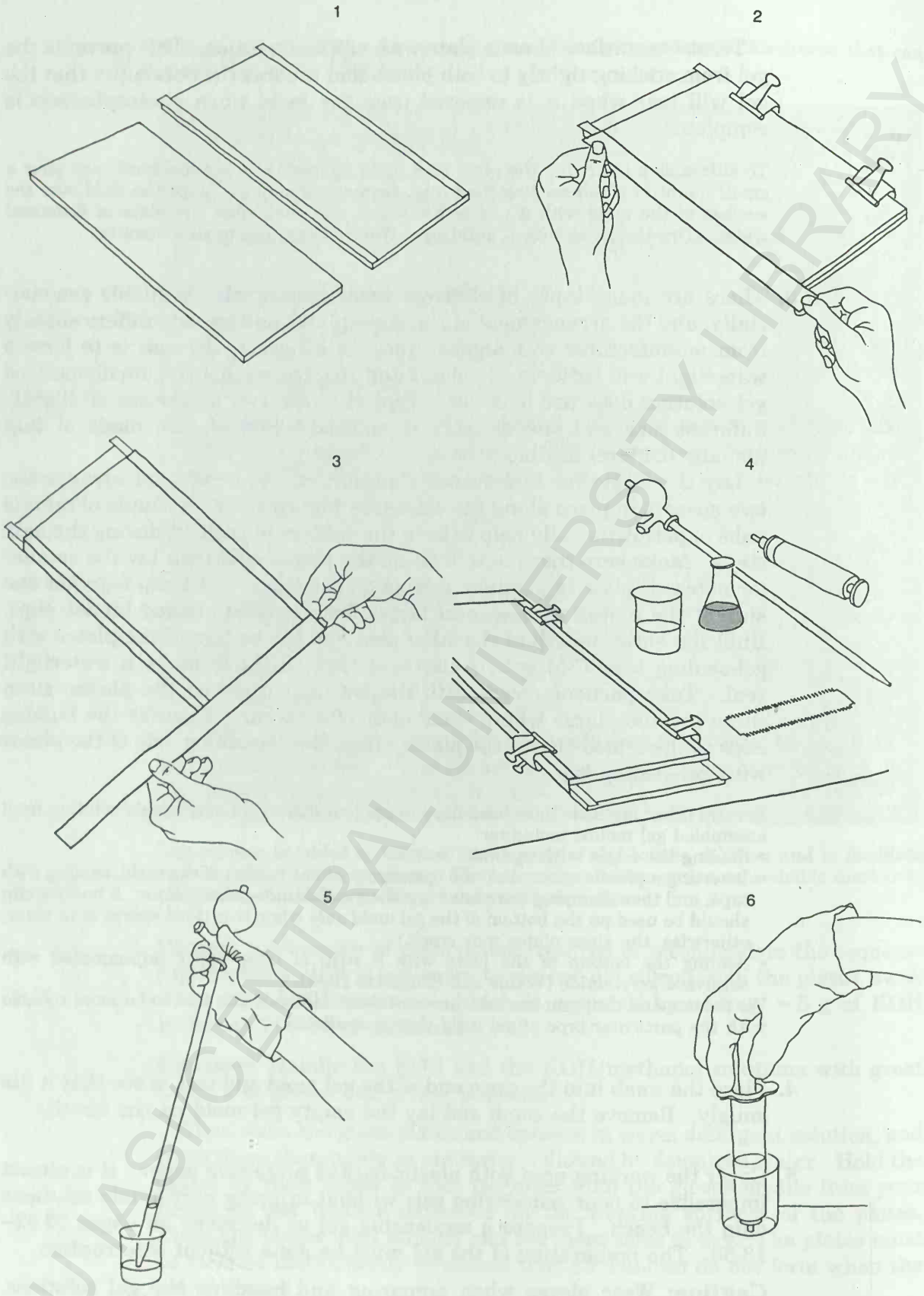

FIGURE 13.8
Preparation of a sequencing gel.

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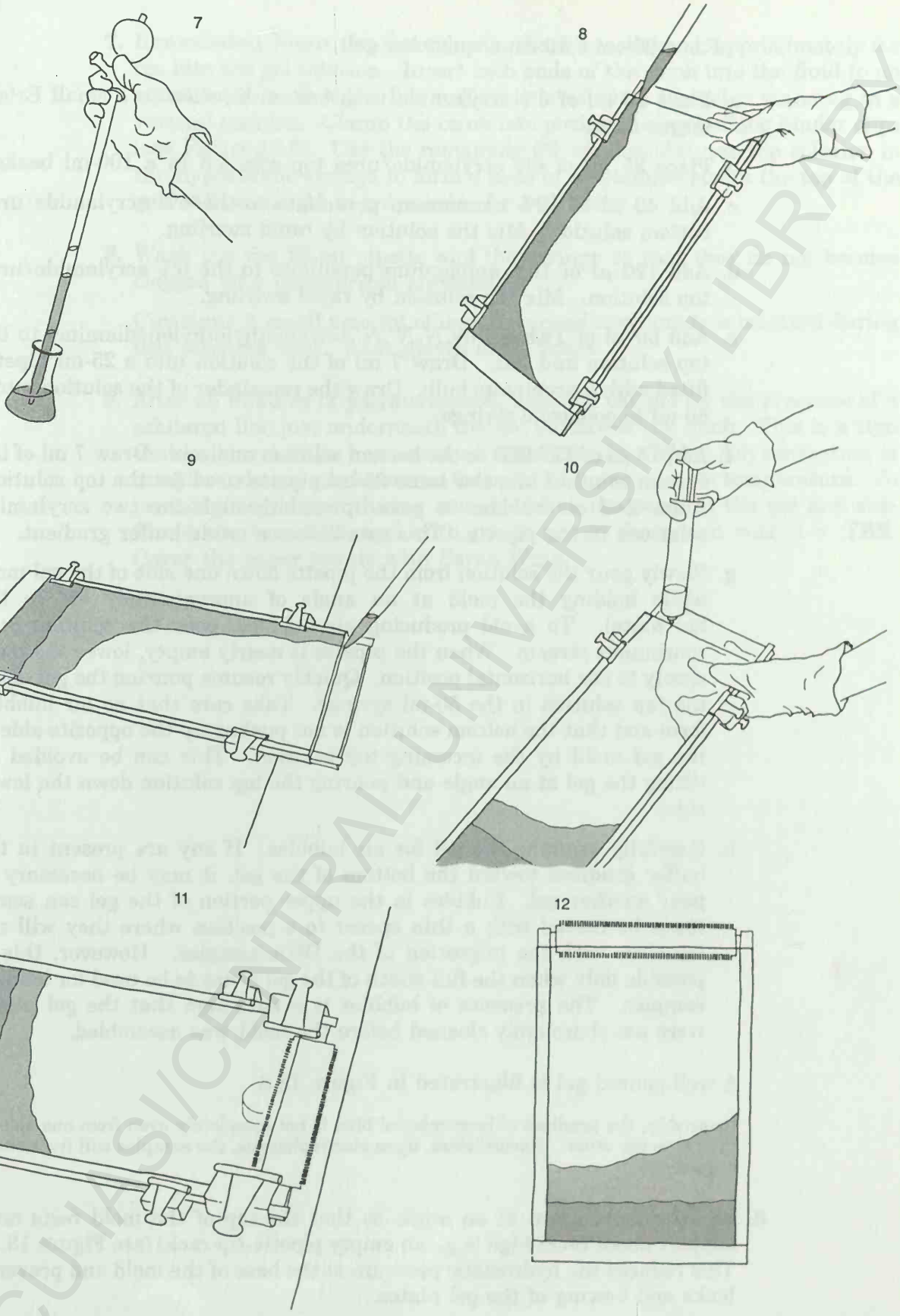

FIGURE 13.8 *(continued)*

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For a 20-cm × 40-cm sequencing gel:

a. Place 10 ml of 6% acrylamide/urea bottom solution in a small Erlenmeyer flask.

b. Place 35 ml of 6% acrylamide/urea top solution in a 100-ml beaker.

c. Add 40 μl of 10% ammonium persulfate to the 6% acrylamide/urea bottom solution. Mix the solution by rapid swirling.

d. Add 120 μl of 10% ammonium persulfate to the 6% acrylamide/urea top solution. Mix the solution by rapid swirling.

e. Add 50 μl of TEMED (*N,N,N′,N′*-tetramethylethylenediamine) to the top solution and mix. Draw 7 ml of the solution into a 25-ml pipette fitted with a propipette bulb. Draw the remainder of the solution into a 50-ml hypodermic syringe.

f. Add 15 μl of TEMED to the bottom solution and mix. Draw 7 ml of the bottom solution into the same 25-ml pipette used for the top solution. Allow 2–3 air bubbles to pass upward through the two acrylamide solutions in the pipette. This establishes a crude buffer gradient.

g. Slowly pour the solution from the pipette down one side of the gel mold while holding the mold at an angle of approximately 45° to the horizontal. To avoid producing air bubbles, pour the solution in a continuous stream. When the pipette is nearly empty, lower the mold slowly to the horizontal position. Quickly resume pouring the gel using the top solution in the 50-ml syringe. Take care that no air bubbles form and that the bottom solution is not pushed up the opposite side of the gel mold by the incoming top solution. This can be avoided by tilting the gel at an angle and pouring the top solution down the lower side.

h. Carefully examine the gel for air bubbles. If any are present in the buffer gradient toward the bottom of the gel, it may be necessary to pour another gel. Bubbles in the upper portion of the gel can sometimes be moved with a thin spacer to a position where they will not interfere with the migration of the DNA samples. However, this is possible only when the full width of the gel is not to be used for loading samples. The presence of bubbles is a sure sign that the gel plates were not thoroughly cleaned before the mold was assembled.

A well-poured gel is illustrated in Figure 13.8.

Invariably, the gradient of bromophenol blue is not completely even from one side of the gel to the other. Nevertheless, upon electrophoresis, the samples will fractionate properly.

6. Lay the mold down at an angle so that the top of the mold rests on a support about 5-cm high (e.g., an empty pipette-tip rack) (see Figure 13.8). This reduces the hydrostatic pressure at the base of the mold and prevents leaks and bowing of the gel plates.

7. Immediately insert the *flat* side of a shark's tooth comb approximately 0.5 cm into the gel solution. Insert both ends of the comb into the fluid to an equal depth so that the flat surface is level when the gel is standing in a vertical position. Clamp the comb into position using bulldog binder clips (see Figure 13.8). Use the remaining 6% acrylamide/urea top solution in the hypodermic syringe to form a bead of acrylamide across the top of the gel. Allow the gel to polymerize for 1–2 hours.

8. Wash out the 25-ml pipette and the syringe so that they do not become clogged with polymerized acrylamide.

   Caution: A small amount of unpolymerized acrylamide is released during this washing step. Wear gloves and a mask.

9. After 45 minutes of polymerization, examine the gel for the presence of a schlieren line just underneath the flat surface of the comb. This is a sign that polymerization has occurred satisfactorily. When polymerization is complete, the gel can be stored for up to 24 hours at room temperature. To prevent dehydration during storage, leave the comb in the gel and surround the top of the gel with paper towels dampened with 1 × TBE. Cover the paper towels with Saran Wrap.

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Loading and Running Gradient Sequencing Gels

An oven or a water bath set at 80°C is required to denature the sequencing reactions just before they are loaded on the gel. Check that the temperature of the oven or water bath is correct before starting the following procedure.

1. When polymerization of the gel is complete, use damp paper towels to wipe away any dried polyacrylamide/urea from the outside of the gel mold. Carefully remove the shark's tooth comb from the top of the gel, and strip the electrical tape from the bottom of the gel mold. This is best done by cutting the tape into several segments with a scalpel blade and then removing each segment in turn. Many workers also remove the electrical tape from the sides of the gel. If this is done, transfer the bulldog binder clips from one side of the mold to the other while stripping the tape. This prevents the glass plates from moving.

2. Attach the gel mold to the electrophoresis apparatus with bulldog binder clips or plastic-coated laboratory clamps. Depending on the design of the electrophoresis apparatus, it may be necessary to clamp plastic-covered metal plates to the gel mold to ensure even diffusion of the heat produced during electrophoresis. Fill the top reservoir with 0.5 × TBE and the bottom reservoir with 1 × TBE (see page 13.47 for 5 × TBE stock solution).

3. Remove the cover from the microtiter plate containing the completed sequencing reactions. Incubate the open plate in the oven (or float it in the water bath) for 10 minutes at 80°C.

   Microtiter plates melt when heated to 85°C.

4. While the plate is incubating, fill a 10-ml hypodermic syringe fitted with a 22-gauge needle with 1 × TBE. Squirt the TBE across the submerged flat loading surface of the gel to remove any fragments of urea and polyacrylamide.

5. Reinsert the shark's tooth comb with its teeth just sticking into the loading surface of the gel. Remove the comb, and once again, wash out the slots of the gel with 1 × TBE.

6. Remove the microtiter plate from the 80°C oven or water bath, and immediately transfer it onto packed ice. Keep the plate at 0°C until the samples have been loaded onto the gel. This retards renaturation of the template and radiolabeled strands.

7. Load 1–2 μl of each sequencing reaction onto adjacent slots of the gel. Keep a record of the order of the templates and load the samples in every reaction set in the same order. TCGA is the best order because the two tracks (G and C) that suffer most from abnormal patterns of migration (e.g., compression) are then adjacent to one another and can be easily compared. In addition, if the gel has been loaded in the order TCGA, the sequence of the complementary strand (3′ → 5′) can be read by flipping the

autoradiograph over and reading the gel from the bottom. (The tracks on the flipped autoradiograph are read from left to right and their order is assumed to be TCGA.)

If there are any spare slots at the edge of the gel, load an aliquot of an old sequencing reaction into one of them. This unambiguously distinguishes right from left on the final autoradiograph.

There are several different methods that can be used to load the samples into the wells of the gel. The choice among them is a matter of personal preference.

- *A Hamilton syringe equipped with a 30-gauge needle.* This needle is narrow enough to enter the space between the two glass plates, and the sample can therefore be layered directly on the surface of the gel. Take care not to touch the gel with the needle. The syringe and needle must be washed out with 1 × TBE (which may be taken from the bottom buffer reservoir) between samples. Excess TBE should be ejected before the next sample is drawn into the syringe and needle.
- *An automatic micropipettor (20 μl) equipped with a standard tip.* Because the tip is too large to fit between the glass plates, the sample must be expelled while the tip is resting on the sill formed by the lower (back) plate, just above the well. The tip need not be washed out between samples as long as no electrophoresis buffer becomes trapped in the tip.
- *A drawn-out glass capillary attached to a mouthpiece that is equipped with a barrier to prevent the sample from being drawn into the user's mouth.* The capillary must be washed out with 1 × TBE between samples. Excess TBE should be blown from the capillary before the next sample of DNA is loaded.

8. When all of the samples are loaded, connect the electrodes to the power pack and the electrophoresis apparatus (anode to the bottom reservoir). The gel should be run at constant power (35–40 W for a 20-cm × 40-cm gel; ~1700 V) for the time required to achieve optimal resolution of the sequence of interest. The time required can be estimated by monitoring the migration of the marker dyes in the formamide/EDTA/XC/BPB gel-loading buffer (see Table 13.3).

   Depending on the distance between the sequence of interest and the oligonucleotide primer, a second loading of the sequencing samples can be applied to the gel approximately 15 minutes after the bromophenol blue from the first loading buffer has migrated from the gradient gel (~3 hours). The sequence obtained from the first loading will be more distal to the primer, whereas that obtained from the second loading will be more proximal. Samples used for the second loading should be denatured at 80°C for 2–3 minutes just before loading.

TABLE 13.3 Migration Rates of Marker Dyes through Denaturing Polyacrylamide Gels

% Polyacrylamide	Bromophenol blue[a]	Xylene cyanol FF[a]
5	35	130
6	26	106
8	19	76
10	12	55
20	8	28

[a] The numbers are the approximate sizes of DNA (in nucleotides) with which the marker dyes will comigrate.

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Autoradiography of Sequencing Gels

1. At the end of the electrophoresis run, turn off the power and disconnect the sequencing apparatus from the power pack. Dispose of the electrophoresis buffer, and then remove the gel mold from the apparatus.

2. Lay the gel mold flat on plastic-backed protective bench paper with the smaller (notched) plate uppermost. Remove any remaining pieces of electrical tape. Using the end of a metal spatula, slowly pry apart the plates of the mold. With luck, the gel will remain attached to the lower plate. If it does not, place the plate back on the gel, invert the plates, and try again.

3. When the glass plates have been separated, cut off the bottom corner of the side of the gel that was loaded first. This serves to orient the gel during the subsequent manipulations. Transfer the gel (together with its supporting plate) to a shallow bath containing 10% methanol and 10% acetic acid in water. This fixes the gel and removes the urea and sucrose, which otherwise prevent the gel from drying completely and causes it to stick to the autoradiographic film. There is no need to agitate the fluid while the gel is being fixed.

   The time required for fixation varies according to the thickness of the gel:

Thickness of gel (mm)	Fixation time (minutes)
0.2	5
0.4	15
0.6	40

   The same batch of fixation fluid can be used to fix several gels.

   If the gel shows signs of detaching from the supporting glass plate, cover it with a piece of stiff plastic netting (available from many hardware stores) to prevent it from escaping and forming a crumpled mass.

4. Pick up the supporting plate by its edges, and lift the gel out of the fixation fluid. Take care that the gel does not slide off the plate. When the plate is clear of the fixation fluid, hold the gel in place with a gloved hand and tilt the plate to allow excess fixation fluid to drain away.

5. Lay the plate (gel uppermost) on a piece of protective paper. Remove any folds or distortions by gently kneading the gel with gloved fingers. Wipe excess fixation fluid from the glass plate with Kimwipes. Try not to touch the surface of the gel with the Kimwipes.

6. Place a piece of Whatman 3MM paper on top of the gel. The paper should be slightly larger (2–3 cm) than the gel in both length and width and should be centered over the gel. Apply gentle pressure so that the gel becomes firmly attached to the rough surface of the paper. Hold the paper in place with one hand and pick up the supporting glass plate with the other. Quickly flip the plate over, and lay it on a dry piece of protective paper.

7. Slide the piece of protective paper to the edge of the bench. Take hold of the leading edge of the 3MM paper and pull it downward while moving the glass plate slowly toward the edge of the bench. The gel will stick to the 3MM paper as it is peeled from the glass plate,

8. Lay the 3MM paper (gel uppermost) on two other pieces of 3MM paper of the same size. Cut a piece of Saran Wrap slightly longer and wider than the gel and lay it on top of the gel. Try to avoid creases and bubbles. This is best done with the help of another person. Hold the corners of the Saran Wrap and pull outward so that it is tightly stretched. Lower the stretched Saran Wrap onto the surface of the gel. Once the Saran Wrap has touched the gel, do not attempt to remove it, since this will cause the gel to tear.

9. Using a paper cutter, trim all three pieces of 3MM paper and the Saran Wrap so that they are nearly the same size as the gel.

10. Dry the gel for 30–40 minutes under vacuum on a commercial gel dryer set at 80°C.

11. Remove the gel from the dryer and peel off the Saran Wrap. The dried gel should feel smooth to the touch but not sticky. To orient the autoradiograph, attach a small adhesive label marked with radioactive ink to the 3MM paper in the space created by cutting the bottom corner of the gel (step 3).

    Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black ink. We find it convenient to make the ink in three grades: very hot (> 2000 cps on a hand-held minimonitor), hot (> 500 cps on a hand-held minimonitor), and cool (> 50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive label. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

12. Establish an autoradiograph by exposing the gel to X-ray film (Kodak XAR-2, XAR-5, or equivalent) for 16–24 hours at room temperature. If possible, use spring-loaded metal cassettes to ensure direct contact between the entire surface of the dried gel and the film emulsion.

13. Develop the autoradiograph, and read the sequence of the DNA as described on page 13.58.

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Reading the Sequence

Reading sequences from gels is not as easy as it looks and is an acquired skill. The following list of tips may help to simplify the process and minimize problems.

1. As soon as the autoradiograph is developed, label it with the date and the names of the templates. Mark each set of sequencing reactions clearly.
2. Be sure that you can distinguish the left and the right sides of the gel. The image of the radioactive ink should appear at the bottom of the sequencing reaction that was loaded on the first track of the gel.
3. When searching for correspondence between the new sequence and one that is already known, look for obvious "signatures" such as homopolymeric runs (e.g., consecutive T residues) or alternating purine and pyrimidines (e.g., GTGTGT). Once found, these signatures can be used to locate the sequence of interest quickly.
4. An unknown sequence should be read and recorded at least twice, preferably by different people. The two readings should then be compared and discrepancies should be resolved, if necessary, by further sequencing.
5. If the gel has been loaded in the order TCGA, the sequence of the complementary strand ($3' \rightarrow 5'$) can be read by flipping the autoradiograph over and reading the gel from the bottom. (The tracks on the flipped autoradiograph are read from left to right and their order is assumed to be TCGA.)
6. The following guidelines are useful when reading gels:
   - Single C bands are generally weaker than single bands of the three other nucleotides.
   - The first A in a homopolymeric run of As is generally stronger than the rest.
   - The first C in a homopolymeric run of Cs is usually much weaker than the second.
   - G bands are weak when they are preceded by a T.

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DIDEOXY-MEDIATED SEQUENCING REACTIONS USING THE KLENOW FRAGMENT OF E. coli DNA POLYMERASE I

Preparation

1. Prepare oligonucleotide primers (0.3 pmole/μl $\cong$ 2 ng/μl) and single-stranded DNA templates (0.1–0.5 μg/μl) as described on page 13.42.

2. Prepare working solutions of dNTPs and ddNTPs. We recommend making up three sets of working solutions:

   - A chase solution that contains all four dNTPs, each at a concentration of 0.5 mM. Dispense the chase solution into aliquots and store them at −70°C in tightly closed microfuge tubes.
   - A set that consists of working solutions of dTTP, dCTP, and dGTP. Each of these solutions contains high concentrations of two of these dNTPs and a lower concentration of one of these dNTPs. The names of these three working solutions identify the particular dNTP that is present in low concentration. For example, the working solution of dCTP contains a lower concentration of dCTP than of the other two dNTPs.
   - A set that consists of four working solutions of ddNTPs, each of which contains a single ddNTP.

   Before use, the appropriate amounts of the dNTP and ddNTP working solutions are mixed together to generate four chain-extension/chain-termination mixtures. For example, the working solution of ddCTP is added to the working solution of dCTP. By varying the relative amounts of the two working solutions in the mixture, it is possible to alter the balance between chain extension and chain termination to suit the particular sequencing task at hand.

   Working solutions of dNTPs and ddNTPs are prepared as described on page 13.60.

3. Pour the required number of polyacrylamide sequencing gels as described on pages 13.45–13.54.

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PREPARATION OF WORKING SOLUTIONS OF dNTPs

1. Prepare working solutions of dNTPs by mixing the four 0.5 mM stock solutions of dNTPs (see page 13.44) in the ratios shown below. For example, the working solution of dTTP is prepared by mixing 2 ml of 0.5 mM dCTP, 2 ml of 0.5 mM dGTP, 0.1 ml of 0.5 mM dATP, and 0.1 ml of 0.5 mM dTTP.

dNTP working solution	0.5 mM Stock solutions of dNTPs (ratio)			
	dTTP	dCTP	dGTP	dATP
dTTP	1	20	20	1
dCTP	20	1	20	1
dGTP	20	20	1	1

It is not necessary to make a working solution containing high concentrations of dATP, which is supplied to the reaction as [^{35}S]dATP.

2. Dispense the working solutions of dNTPs into aliquots and store them at −70°C in tightly closed microfuge tubes.

PREPARATION OF WORKING SOLUTIONS OF ddNTPs

1. Prepare working solutions of ddNTPs by diluting the four 10 mM stock solutions of ddNTPs (see page 13.44) with TE (pH 8.0) and water as shown below.

ddNTP working solution	10 mM ddNTP (µl)	TE (pH 8.0) (µl)	H_2O (µl)
ddTTP	60	40	900
ddCTP	8	92	900
ddGTP	20	80	900
ddATP	9	91	900

2. Dispense the working solutions of ddNTPs into aliquots and store them at −70°C in tightly closed microfuge tubes.

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Sequencing Reactions

1. If N different templates of DNA are to be sequenced, add to a microfuge tube:

primer (0.3 pmole ≅ 2 ng = 1 μl)	$N + 1$ μl
Tris-Mg solution	$N + 1$ μl
H_2O	$7N + 7$ μl

The final number of microliters of annealing mixture in the tube should equal $9N + 9$.

Tris-Mg solution

100 mM Tris · Cl (pH 8.5)
100 mM $MgCl_2$

Separate annealing mixtures should be prepared for each primer that is used.

2. Place 9 μl of annealing mixture in a 450-μl microfuge tube, and then add 2 μl of single-stranded DNA template. Close the top of the tube, and incubate it for 30 minutes at 55°C.

The concentration of single-stranded DNA in small-scale preparations of bacteriophage M13 recombinants varies from 0.05 μg/μl to 0.5 μg/μl, depending on the growth rate of the particular bacteriophage. Under the conditions normally used for DNA sequencing, the template DNA is present in excess. Small variations in the amount of template added to different sets of reactions therefore do not generally affect the quality of the results (however, see the Troubleshooting Guide, Table 13.5, pages 13.76–13.77).

3. While the primer and template are annealing at 55°C, thaw

the dNTP working solutions
the ddNTP working solutions
[^{35}S]dATP (600 Ci/mmole; 10 mCi/ml in water)

Store the thawed solutions on ice.

4. Make up four chain-extension/chain-termination mixtures as follows:

a. Label four microfuge tubes ddA, ddC, ddT, and ddG.

b. For each template to be sequenced, add to the appropriately labeled tube:

the appropriate ddNTP working solution	2.5 μl
the appropriate dNTP working solution	7.5 μl
[^{35}S]dATP (4 μCi)	0.4 μl

The total number of microliters in each tube should equal 10.4 times the number of templates.

5. In a fresh microfuge tube, make up a Klenow mixture. For each template to be sequenced, mix:

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10 mM Tris · Cl (pH 8.0)	8 μl
0.1 M dithiothreitol	1 μl

The total number of microliters in the tube should equal 9 times the number of templates. Store the Klenow mixture at room temperature.

6. At the end of the incubation (step 2), remove the primer-template mixture from the 55°C incubator. Centrifuge the tube briefly in a microfuge to return any condensation to the bottom of the tube.

   The primer-template mixture can be stored at −20°C at this stage, if desired.

7. For each template, designate a group of four contiguous wells (labeled T, C, G, A) in a 96-well disposable microtiter plate (e.g., Baxter, B1190-17) (see Figure 13.9).

   Using an automatic pipettor, transfer 4 μl of the annealed primer-template mixture to the bottom of each of these four U-shaped wells. Continue in the same fashion until all of the annealed template-primers have been dispensed into wells.

8. Dispense 2 μl of the appropriate chain-extension/chain-termination mixture prepared in step 4 into each well. The well designated A should receive the ddA mixture; the well marked C should receive the ddC mixture, and so on. The fluid should be deposited as a droplet on the wall of each well, near the rim.

   The most accurate and rapid device for transferring the chain-extension/chain-termination mixtures to each well is a Hamilton repeating dispenser (model PB-600-1; Baxter B6732-11) and a 100-μl gas-tight syringe (model 1710-TLL; Baxter S9662-81) fitted with a Luer-lock adapter (Baxter B6732-21) that will accept standard disposable pipette tips. Use a fresh tip for each chain-extension/chain-termination mixture. Work quickly to avoid problems caused by evaporation.

9. Add the Klenow fragment of *E. coli* DNA polymerase I (6 units for each template sequence; i.e., 1.5 units for each of four sequencing reactions) to the Klenow mixture prepared in step 5. Mix the contents of the tube thoroughly by gently tapping the side of the tube with a finger.

10. Using a Hamilton repeating dispenser (see note to step 8), immediately dispense 2 μl of the Klenow reaction mixture into each well. The fluid should be deposited as a droplet on the wall of each well, near the rim. Work quickly to avoid problems caused by evaporation. When all of the wells have received the Klenow reaction mixture, cover the plate with a plastic lid.

    To prevent accidental transfer of fluid from one well to another, the lid may be lined with Whatman 3MM paper.

11. To mix the reagents and start the chain-extension/chain-termination reaction, centrifuge the droplets to the bottom of the U-shaped wells. Place the covered microtiter plate in a suitable holder (e.g., Sorvall PN11065) in a swing-out centrifuge. Turn on the centrifuge at maximum acceleration. When the rotor speed reaches 2000 rpm, immediately turn off the centrifuge.

12. Remove the plate from the centrifuge, and incubate the reactions for 15 minutes at room temperature.

13. While the reactions are incubating, thaw the chase solution which contains all four dNTPs, each at a concentration of 0.5 mM.

    In most cases, there is no need to add additional Klenow enzyme to the reaction during the chase period. However, if there is a persistent problem with bands appearing in all four tracks, a more elaborate chase solution should be used containing all four dNTPs (each at a concentration of 0.5 mM), 10 mM Tris·Cl (pH 8.5), 10 mM $MgCl_2$, and the Klenow fragment of *E. coli* DNA polymerase I (1 unit per 8 μl of chase solution).

14. After 12 minutes of incubation, begin adding the chase solution (2 μl) to the wall of each well. After a total of 15 minutes of incubation, centrifuge the microtiter plate briefly as described in step 11 to mix the solutions. Continue incubation for a further 12 minutes at room temperature.

15. After a total of 27 minutes of incubation, begin adding formamide/EDTA/XC/BPB gel-loading buffer (4 μl) to the wall of each well. After a total of 30 minutes of incubation, centrifuge the plate as described in step 11 to mix the solutions. The plate may be covered with a lid, sealed with Parafilm, and stored overnight at −20°C.

Formamide/EDTA/XC/BPB gel-loading buffer

formamide	10 ml
xylene cyanol FF	10 mg
bromophenol blue	10 mg
0.5 M EDTA (pH 8.0)	200 μl

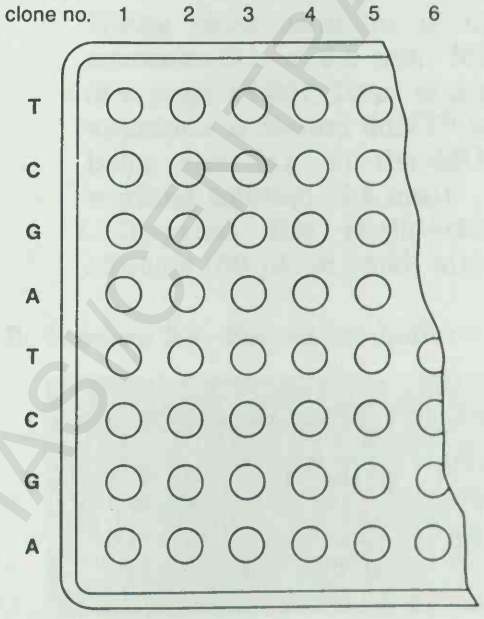

FIGURE 13.9
Microtiter plate.

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Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

Several companies sell formamide distilled and packaged under nitrogen that need not be purified before use (e.g., BRL 5515UA).

16. Separate the products of the sequencing reactions by electrophoresis through denaturing polyacrylamide gels as described on pages 13.45–13.58.

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DIDEOXY-MEDIATED SEQUENCING REACTIONS USING SEQUENASES

Sequencing with Sequenase or Sequenase version 2.0 is carried out in two steps:

- A brief polymerization reaction in which limiting concentrations of dNTPs (including one radiolabeled dNTP) are used to extend the primer by an average of 25 nucleotides

- A set of four chain-extension/chain-termination reactions in which the radiolabeled chains are rapidly extended and then terminated by incorporation of a ddNTP

Preparation

1. Prepare oligonucleotide primers (0.3 pmole/μl $\cong$ 2 ng/μl) and single-stranded DNA templates (0.1–0.5 μg/μl) as described on page 13.42.

2. Prepare the following reaction mixtures from stock solutions of dNTPs (0.5 mM) and ddNTPs (10 mM) (see page 13.44):

   a. *Stock labeling mixture*

dGTP (0.5 mM)	15 μl
dCTP (0.5 mM)	15 μl
dTTP (0.5 mM)	15 μl
deionized H_2O to a final volume of 1 ml	

   Dispense the stock labeling mixture into aliquots (20 μl) in small microfuge tubes and store them at −20°C.

   b. *Chain-extension/chain-termination mixtures*

   Dilute stock solutions of ddNTPs (10 mM) with water to a final concentration of 0.5 mM. Mix 160 μl of *each* dNTP working solution (see page 13.60), 10 μl of 5 M NaCl, 334 μl of H_2O, and 16 μl of the appropriate diluted ddNTP working solution (0.5 mM) for the mixture being made (e.g., for the ddG mixture, use 16 μl of the diluted ddGTP working solution [0.5 mM]).

   Dispense the chain-extension/chain-termination mixtures into aliquots (50 μl) in small microfuge tubes and store them at −20°C.

3. Prepare 5 × Sequenase buffer.

5 × Sequenase buffer

1 M Tris · Cl (pH 7.5)	200 μl
1 M $MgCl_2$	100 μl
5 M NaCl	25 μl
H_2O to 1 ml	

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4. Have available a fresh solution of 0.1 M dithiothreitol, TE (pH 7.5) and water baths or incubators set at 37°C and 55°C.

5. Pour the required number of polyacrylamide sequencing gels as described on pages 13.45–13.54.

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Sequencing Reactions

1. If N different templates of DNA are to be sequenced, add to a microfuge tube:

primer (0.5 pmole)	$2.5N + 2.5$ μl
5× Sequenase buffer	$2.0N + 2.0$ μl
H_2O	$4.5N + 4.5$ μl

   The total number of microliters of annealing mixture in the tube should equal $9N + 9$.

2. Place 9 μl of annealing mixture in a 450-μl microfuge tube, and then add 2 μl of single-stranded DNA template. Close the top of the tube and incubate it for 30 minutes at 55°C.

   The concentration of single-stranded DNA in small-scale preparations of bacteriophage M13 recombinants varies from 0.05 μg/μl to 0.5 μg/μl, depending on the growth rate of the particular bacteriophage. Under the conditions normally used for DNA sequencing, the template DNA is present in excess. Small variations in the amount of template added to different sets of reactions therefore do not generally affect the quality of the results (however, see the Troubleshooting Guide, Table 13.5, pages 13.76–13.77).

3. While the primer and template are annealing at 55°C, thaw:

   the stock labeling mixture (see page 13.65)
   the chain-extension/chain-termination mixtures (see page 13.65)
   [^{35}S]dATP (600 Ci/mmole; 10 mCi/ml in water)

   Store the thawed solutions on ice.

   The reaction conditions have been optimized to obtain the sequence of DNA lying up to 300–400 nucleotides from the 5′ terminus of the primer. Reading of sequences closer to or further away from the primer can be facilitated by the modifications presented in Table 13.4.

TABLE 13.4 Modification of Sequenase Reactions to Optimize Sequencing of Different Lengths of DNA

Distance of target DNA from 5′ terminus of primer (nucleotides)	Modification
20–100	Dilute the labeling mixture tenfold more. Halve the time of incubation of both the labeling and chain-extension/chain-termination reactions.
100–350	Normal reaction conditions.
>350	Increase the concentrations of unlabeled dNTPs and [^{35}S]dATP in the labeling reaction fourfold. Double the time of incubation of the labeling reaction.

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4. After the primer and template have been incubating for 25 minutes, prepare the Sequenase/[^{35}S]dATP mixture. For each DNA template that is to be sequenced, mix:

stock labeling mixture	0.5 μl
TE (pH 7.4)	8.0 μl
[^{35}S]dATP (600 Ci/mmole; 10 mCi/ml in water)	0.5 μl
0.1 M dithiothreitol	0.5 μl
Sequenase (or Sequenase version 2.0)	0.5 μl

The total number of microliters in the enzyme/[^{35}S]dATP mixture should be 10 times the number of DNA templates to be sequenced. Store the tube on ice.

Because the specific activities of Sequenase and Sequenase version 2.0 are different from each other, the manufacturer (United States Biochemical) uses different definitions for the units of activity of the two enzymes. Approximately 2.5 units of Sequenase are equivalent to 1 unit of Sequenase version 2.0. Both enzymes are supplied by the manufacturer at a concentration that generally is optimal for sequencing after dilution by a factor of 8- to 20-fold. Dilution is required to reduce the concentration of glycerol, which can cause distortions in the region of the sequencing gel that contains fragments of DNA in the 400–600-nucleotide range.

5. After 30 minutes of incubation, remove the primer-template mixture from the 55°C incubator. Centrifuge the tube briefly in a microfuge to return any condensation to the bottom of the tube.

The primer-template mixture can be stored at −20°C at this stage, if desired.

6. For each template, designate a group of four contiguous wells (labeled T, C, G, A) in a 96-well disposable microtiter plate (e.g., Baxter B1190-17) (see Figure 13.9, page 13.63).

Using an automatic pipettor, transfer 4 μl of the annealed primer-template mixture to the bottom of each of these four U-shaped wells. Continue in the same fashion until all of the annealed template-primers have been dispensed into wells.

7. Transfer 2 μl of the enzyme/[^{35}S]dATP mixture to the side of each well. The fluid should be deposited as a droplet on the wall of each well, near the rim. Work quickly to avoid problems caused by evaporation. When all of the wells have received the enzyme/[^{35}S]dATP mixture, cover the plate with a plastic lid.

8. To mix the reagents and start the labeling reaction, centrifuge the droplets to the bottom of the U-shaped wells. Place the covered microtiter plate in a suitable holder (e.g., Sorvall PN11065) in a swing-out centrifuge. Turn on the centrifuge at maximum acceleration. When the rotor speed reaches 2000 rpm, immediately turn off the centrifuge.

9. Incubate the plate for 10 minutes at room temperature.

10. Dispense 2 μl of the appropriate chain-extension/chain-termination mixture into each well. The fluid should be deposited as a droplet on the

wall of each well, near the rim. The well designated A should receive the ddA mixture; the well marked C should receive the ddC mixture, and so on. When all of the wells have received the appropriate chain-extension/chain-termination mixture, cover the plate with a plastic lid.

The most accurate and rapid device for transferring the chain-extension/chain-termination mixtures to each well is a Hamilton repeating dispenser (model PB-600-1; Baxter B6732-11) and a 100-μl gas-tight syringe (model 1710-TLL; Baxter S9662-81) fitted with a Luer-lock adapter (Baxter B6732-21) that will accept standard disposable pipette tips. Use a fresh tip for each chain-extension/chain-termination mixture. Work quickly to avoid problems caused by evaporation.

To prevent accidental transfer of fluid from one well to another, the lid may be lined with Whatman 3MM paper.

11. To mix the reagents and start the chain-extension/chain-termination reaction, centrifuge the droplets to the bottom of the U-shaped wells as described in step 8.

12. Incubate the plate for 10 minutes at 37°C.

13. Add formamide/EDTA/XC/BPB gel-loading buffer (4 μl) to the wall of each well. Centrifuge the plate as described in step 8 to mix the solutions.

The plate may be covered with a plastic lid, sealed with Parafilm, and stored overnight at −20°C.

Formamide/EDTA/XC/BPB gel-loading buffer

formamide	10 ml
xylene cyanol FF	10 mg
bromophenol blue	10 mg
0.5 M EDTA (pH 8.0)	200 μl

For preparation of deionized formamide, see page 13.64

14. Separate the products of the sequencing reactions by electrophoresis through denaturing polyacrylamide gels as described on pages 13.45–13.58.

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SEQUENCING DENATURED DOUBLE-STRANDED DNA TEMPLATES

Dideoxy-mediated sequencing can be performed on denatured plasmid (for recent references, see Chen and Seeburg 1985; Hattori and Sakaki 1986; Mierendorf and Pfeffer 1987) or bacteriophage λ templates (Mehra et al. 1986) using oligonucleotide primers that are complementary to sequences flanking restriction sites commonly used for cloning. Frequently, these primers are longer (25–29 nucleotides) than primers normally used for sequencing single-stranded templates. Longer primers give rise to fewer artifactual bands when used with denatured double-stranded DNA templates such as bacteriophage λ or plasmids.

Until recently, the quality of the sequence obtained from such templates left much to be desired. However, if care is taken with the preparation of the template, it is now possible to obtain up to 200 nucleotides of virgin sequence per reaction set. The best results are obtained using plasmid DNA that has been purified by equilibrium centrifugation in CsCl–ethidium bromide gradients. Large-scale preparations of plasmid DNA purified by precipitation with polyethylene glycol also give acceptable results, although less reproducibly. Minipreparations of plasmid DNA must be treated with RNAase or LiCl to remove contaminating small fragments of RNA that can serve as primers in the sequencing reaction. This improves the quality of the sequence to the point where it is usually possible to verify a construct or to confirm the site of a mutation. Large-scale sequencing of virgin DNA is, however, not yet advisable.

The Klenow fragment of *E. coli* DNA polymerase I is sensitive to contaminants in many plasmid preparations and is unable to extend growing chains for long distances. The resulting elevated level of nonspecific termination leads to the appearance of artifactual bands in all lanes of the sequencing gel. Reverse transcriptase is slightly better, but it is not as good as either version of Sequenase for the sequencing of denatured double-stranded DNA templates. Below we outline the use of Sequenase to obtain nucleotide sequence from large-scale preparations of plasmids purified by equilibrium centrifugation or minipreparations of plasmid DNA that have been treated with RNAase or LiCl. The method can be easily adapted to sequencing DNAs cloned in bacteriophage λ vectors (see, e.g., Mehra et al. 1986; Hardwick and Huberman 1988).

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Sequencing of Plasmid DNAs Purified by Equilibrium Centrifugation in CsCl–Ethidium Bromide Gradients

This method is a modification of procedures published by Zhang et al. (1988) and by the manufacturer of Sequenase (United States Biochemical).

1. Purify the plasmid DNA from a large-scale culture of bacteria as described in Chapter 1, pages 1.42–1.45. Remove the ethidium bromide by extraction with isoamyl alcohol as described in Appendix E.

2. Add 4 μl of 1 N NaOH to 2.5 μg of superhelical plasmid DNA (in a volume of 16 μl of TE [pH 7.6]). Incubate the solution for 5 minutes at room temperature.

3. Add 2 μl of 5 M ammonium acetate (adjusted to pH 4.6 with acetic acid). Immediately mix the solution by vortexing briefly, and then quickly add 50 μl of ethanol at 0°C. Mix again, and store the solution on ice for 15 minutes.

4. Recover the denatured plasmid DNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Carefully remove the supernatant by gentle aspiration, and then add 1 ml of 70% ethanol (4°C) to the tube. Recentrifuge for a further 2 minutes.

5. Carefully remove and discard the ethanol. Leave the open tube on the bench until the last traces of ethanol have evaporated. Dissolve the DNA in 9 μl of H_2O and store it on ice until needed.

6. Carry out sequencing reactions as described on pages 13.67–13.69 with the following modifications:

   - Use all of the plasmid DNA (2.5 μg) in a single set of sequencing reactions.
   - Titrate the amount of primer. Although denatured double-stranded DNA templates require more primer than single-stranded templates, the addition of too much primer will lead to an unacceptably high level of background. In general, between 1 and 5 pmoles of oligonucleotide primer will be optimal for 2.5 μg of denatured plasmid template.
   - The primer should be complementary to sequences lying 50–100 nucleotides upstream of the target sequence. The average length of sequence that can be determined from denatured double-stranded templates is approximately 250 nucleotides.
   - Anneal the primer and template for 5 minutes at 60°C, and then allow the solution to cool to room temperature for 20–30 minutes before setting up sequencing reactions.
   - Use the standard labeling mixture and the standard chain-extension/chain-termination mixtures. However, the amount of [^{35}S]dATP in the enzyme/[^{35}S]dATP mixture should be increased to 1 μl (10 μCi) per set of four sequencing reactions.

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Note

Target DNAs cloned in bacteriophage λ can be sequenced essentially as described above. However, improved results are obtained if the bacteriophage DNA is cleaved with restriction enzymes that release the target DNA from the vector sequences. The DNA is then purified by extraction with phenol and precipitated with ethanol. Approximately 3 μg of DNA is used for each set of sequencing reactions. If background problems arise, 5′-end-labeled primers can be used for sequencing.

Removal of RNA from Minipreparations of Plasmid DNA by Precipitation with Lithium Chloride

1. Purify plasmid DNA from small-scale bacterial cultures as described on page 13.71.
2. After the last precipitation with ethanol (step 5, page 13.71), redissolve the DNAs in 100 μl of TE (pH 7.6).
3. To each minipreparation of plasmid DNA, add 300 μl of an ice-cold solution of 4 M LiCl. Store the minipreparations for 30 minutes on ice.
4. Centrifuge the plasmid DNAs at 12,000*g* for 10 minutes at 4°C in a microfuge.
5. Transfer the supernatants to fresh microfuge tubes. Add 600 μl of isopropanol. Store the tubes for 30 minutes at room temperature.
6. Recover the plasmid DNAs by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge.
7. Carefully remove the supernatants by aspiration, and add 1 ml of 70% ethanol to each tube. Vortex the tube briefly, and then recentrifuge at 12,000*g* for 5 minutes at 4°C in a microfuge.
8. Carefully remove the supernatants by aspiration, and leave the open tubes on the bench until the last traces of ethanol have evaporated.
9. Resuspend the pellets in 10 μl of TE (pH 7.6). Check the recovery of plasmid DNAs by analyzing 1 μl of the solution by electrophoresis through a 0.8% agarose gel.
10. Denature the plasmid DNAs as described on page 13.71, and carry out sequencing reactions as described on pages 13.67–13.69.

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PROBLEMS THAT ARISE WITH DIDEOXY-MEDIATED SEQUENCING

Although dideoxy-mediated sequencing usually goes smoothly, it is hardly ever perfect. For example, there is a constant need to adjust the ratio of ddNTPs to dNTPs in the chain-extension/chain-termination reactions to maximize the amount of information that can be obtained. In addition, more serious problems occasionally arise that compromise the extent or quality of the DNA sequence obtained. Most of these problems can be solved by minor adjustments to the techniques used to prepare templates or by small alterations in the conditions used for chain-extension/chain-termination reactions or polyacrylamide gel electrophoresis. An important first step is to determine whether the problem is systematic (i.e., affects all sequencing reactions) or whether it is template-specific. If this is not immediately obvious from a comparison of the results obtained from different templates that were sequenced on the same day, it can be rapidly ascertained by carrying out sequencing reactions with a reliable template (i.e., one that has given good results previously or one that is supplied as part of a commercially produced sequencing kit).

Template-specific Problems

The vast majority of template-specific problems have one of the four causes listed below. The diagnostic symptoms of each of these problems and possible suggestions for methods to solve them are listed in Table 13.5.

1. The presence of contaminating DNA (usually bacterial chromosomal DNA) in the preparation.
2. The presence of residual polyethylene glycol or high concentrations of EDTA in the preparation.
3. Low concentrations of template DNA in the chain-extension/chain-termination reactions.
4. The absence of a primer-binding site. This problem arises chiefly when sequencing deletion mutants generated by digestion with exonuclease III or BAL 31. Occasionally, these deletions extend back into the primer-binding region, thereby eliminating the ability to obtain DNA sequence. This problem can sometimes be corrected by using a primer complementary to sequences that lie further upstream of the target DNA.

Systematic Problems

Systematic problems with chain-extension/chain-termination reactions can, in theory, be due to deficiencies in any of the components in the reactions (primer, dNTPs, ddNTPs, [^{35}S]dATP, etc.). In practice, however, the most common cause of difficulty is the DNA polymerase, particularly when sequencing is carried out with the Klenow fragment of *E. coli* DNA polymerase I. This enzyme needs to be handled with great care. It should never be vortexed, and it should be kept cold at all times. The enzyme should be diluted immediately before use and should not be stored in a diluted state. The Klenow fragment expressed from a cloned gene, which lacks all 5′→3′

exonuclease activity, is superior to the fragment produced by subtilisin treatment of *E. coli* DNA polymerase I.

The most common types of systematic sequencing problems are listed in Table 13.5 together with suggestions for solving them. When problems of this type arise, it is often useful to use the reagents provided in commercial sequencing kits to identify the defective component of the chain-extension/chain-termination reaction.

Problems with Polyacrylamide Gels

Problems with polyacrylamide gels are of two types: (1) technical difficulties arising from the use of poor reagents (see Table 13.5) and, more rarely, (2) problems caused by secondary structure within the template or radiolabeled product. Problems due to secondary structure appear in two forms:

- *Blocks* caused by regions of secondary structure (e.g., homopolymeric tracts) in the template strand that severely impede the progress of the DNA polymerase. These blocks can sometimes be relieved by using reverse transcriptase, *Taq* DNA polymerase, or Sequenase instead of the Klenow fragment of *E. coli* DNA polymerase I or by performing the sequencing reactions at 55°C (Gomer and Firtel 1985). In a few cases, however, the blocks and the sequences that lie beyond them can be sequenced only by the Maxam-Gilbert method.
- *Compressions*, in which bands in a particular location within the gel are crowded together, resulting in unreadable sequence. In the region of the gel above the compression, the space between the bands frequently becomes abnormally large. These are gel artifacts caused by short stretches of dyad symmetry—especially those containing a high proportion of G and C residues—at the 3′ terminus of the radiolabeled strand. These regions of ambiguity can often be resolved by sequencing the opposite strand. If the problem persists, try substituting the base analog dITP for dGTP in the chain-extension/chain-termination reaction. This analog forms I-C base pairs that contain only two hydrogen bonds instead of the three normally formed by G-C base pairs. Although the Klenow fragment of *E. coli* DNA polymerase I will accept dITP, Sequenases incorporate the analog more efficiently and are therefore the enzymes of choice when carrying out sequencing reactions with dITP. Because the use of dITP accentuates pauses in the chain-extension/chain-termination reaction, sequencing reactions containing the base analog should always be run in parallel with reactions containing dGTP. The concentrations of dNTPs and ddNTPs in the stock labeling mixture and chain-extension/chain-termination mixtures when dITP is substituted for dGTP are given below.

dITP stock labeling mixture

dITP (0.5 mM)	30 μl
dCTP (0.5 mM)	15 μl
dTTP (0.5 mM)	15 μl
deionized H_2O to a final volume of 1 ml	

Dispense the dITP stock labeling mixture into aliquots in microfuge tubes and store them at −20°C.

ddITP chain-extension/chain-termination mixtures

Dilute stock solutions of ddNTPs (10 mM) (see page 13.44) with water to a final concentration of 0.5 mM. Use the diluted stock solutions of ddNTPs and the 0.5 mM stock solutions of dNTPs (see page 13.44) to make up chain-extension/chain-termination mixtures as shown below. Dispense the ddITP chain-extension/chain-termination mixtures into aliquots in microfuge tubes and store them at −20°C.

ddNTP mixture	0.5 mM Stock solutions (μl)				Diluted working solution of ddNTP[a] (0.5 mM)(μl)	5 M NaCl (μl)	H_2O (μl)
	dITP	dATP	dTTP	dCTP			
ddI	320	160	160	160	3	10	187
ddA	320	160	160	160	16	10	174
ddT	320	160	160	160	16	10	174
ddC	320	160	160	160	16	10	174

[a] Use the appropriate ddNTP for the mixture being made; for example, for the ddI mixture, use 3 μl of the diluted working solution of ddITP (0.5 mM).

The quality of sequence obtained is never as good when base analogs are used instead of conventional dNTPs. It is therefore helpful to load each set of four sequencing reactions into eight adjacent lanes of a polyacrylamide gel in the order IATCITAC. This ensures that each of the four sequencing reactions is adjacent to the other three and allows the order of closely spaced bands to be determined more easily.

Compressions not resolved by dITP can occasionally be resolved by using 7-deaza-dGTP (Mizusawa et al. 1986). However, dITP is usually the more effective base analog and is therefore the first choice for resolving compressions. If problems persist, the addition to the chain-extension/chain-termination reactions of 0.5 μg of single-stranded DNA-binding protein from *E. coli* usually eliminates the difficulty. When using single-stranded DNA-binding protein (United States Biochemical 70032), it is necessary to treat the reactions with proteinase K (0.1 μg/reaction) for 20 minutes at 65°C after adding the formamide/EDTA/XC/BPB gel-loading buffer. This allows the DNA to enter the sequencing gel and prevents smearing of bands.

The substitution of dITP for dGTP causes nucleic acids to migrate slightly faster through denaturing polyacrylamide gels.

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TABLE 13.5 Troubleshooting Guide

Problem	Potential causes	Possible solutions
Entire film blank or nearly so	Essential component (enzyme, dNTPs, primer, radioactive precursor) omitted from reaction Saran Wrap not removed from gel after drying	Check that the correct labeled dNTP was used Make a new batch of labeling and chain-extension/chain-termination mixtures Use a new batch of [^{35}S]dATP
One set of sequencing reactions yields four blank tracks	No template DNA Primer-binding site deleted from template High concentrations of EDTA present Incorrect primer used	Check concentration of template on agarose gel Repeat sequencing reactions after reprecipitating the template DNA with ethanol Resynthesize primer Check that the primer does not display a high degree of self-complementarity If tracks are still blank, abandon this particular template
All sequencing reactions on gel contain the same blank track	Problems with a specific chain-extension/chain-termination mixture	Make a fresh batch of chain-extension/chain-termination mixture
Variation in incorporation of radiolabel from template to template	Variation in template concentration Dirty templates	Check concentrations of template DNAs on agarose gels; make sure all sequencing reactions contain approximately equal amounts of template Purify a new batch of template DNAs
Generalized high background in all four lanes makes sequence difficult to read	Dirty templates, perhaps contaminated by chromosomal DNA	Replate recombinant bacteriophage on a fresh culture of indicator bacteria grown from a single colony; make sure that single-stranded DNA is prepared from an infected culture grown for not more than 4.5 hours
Shadow bands or double bands present in one set of sequencing reactions	More than one template present Primer is binding to more than one site Heterogeneity in primer length	Plaque-purify recombinant bacteriophage; make sure that single-stranded DNA is prepared from an infected culture grown for not more than 4.5 hours Titrate primer or use an alternative primer Repurify primer
Shadow bands in all sequencing reactions	Primer concentration is too high Klenow fragment is deteriorating or is in limiting concentration Heterogeneity in primer length	Titrate primer Increase amount of Klenow fragment to 2 units per template Add additional Klenow fragment during the chase (see note to step 13, page 13.63) Try a new batch of enzyme (see pages 13.73–13.74 for precautions in handling Klenow fragment) Repurify primer

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Bands in all four tracks of all sequencing reactions	Klenow fragment is badly deteriorated or in limiting concentration Contaminated templates	Increase amount of Klenow fragment to 2 units per template Try a new batch of enzyme (see pages 13.74–13.75 for methods to resolve compressions) Repurify primer
Bands in all four tracks in particular regions of sequence	Homopolymeric tracts in the template, especially Gs	Sequence the complementary strand
Smeary bands	Polyacrylamide gel is defective	Make up new gel mixtures using freshly purified components Make sure that the gel mixture is at room temperature when gel is poured; otherwise, urea may precipitate
Wavy bands	Wells not thoroughly washed before loading	Pour new gel and wash wells thoroughly
Black dots on autoradiograph	Precipitation of TBE or urea in gel mixtures	Make up new gel mixtures using freshly purified components Make sure that the gel mixture is at room temperature when gel is poured; otherwise, urea may precipitate
Radioactivity remains in well	Samples not properly denatured	Transfer samples to microfuge tubes and denature by boiling for 5 minutes; chill samples to 0°C and load gel within 20 minutes
Bands are faint at top of gel	Ratio of ddNTP to dNTP is too high	Adjust ratio of ddNTP to dNTP
Bands are faint at bottom of gel	Ratio of ddNTP to dNTP is too low	Adjust ratio of ddNTP to dNTP
	Sequencing with Sequenases	
Bands are faint at bottom of gel	Concentration of template DNA is limiting, resulting in the synthesis of long DNA molecules during the elongation reaction	Increase the amount of template Use a primer more distant from the target site
Bands are faint at top of gel	Concentration of dNTP, most probably dATP, is limiting	Check that specific activity of [^{35}S]dATP is 600 Ci/mmole Add 1 μl of [^{35}S]dATP to the labeling reaction
Bands in all four tracks of sequencing reactions at bottom of gel	Sequenase is inactivated during elongation reaction	Incubate the elongation reaction for 5 minutes at *20°C* (check temperature!)
Bands in all four tracks of sequencing reactions at top of gel	Termination reaction is working inefficiently	Repurify template DNA Check that termination reaction is carried out at *37°C*

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Sequencing by the Maxam-Gilbert Method

The Maxam-Gilbert method requires that the target DNA be labeled at only one terminus (see Figure 13.2, page 13.12). The sequence of nucleotides lying within 250 nucleotides of the labeled terminus can then be determined by carrying out the five base-specific chemical reactions described on pages 13.88–13.93 and separating the resulting fragments of DNA by denaturing polyacrylamide gel electrophoresis. The target DNA can be of any length and can be radiolabeled in a number of ways, depending on the nature of the termini of the DNA.

Asymmetric Labeling of Target DNA

Double-stranded DNA can be labeled in the following ways:

- At its 5′ termini by bacteriophage T4 polynucleotide kinase and [γ-^{32}P]ATP (see Chapter 11, pages 11.31–11.32) or at its 3′ termini by calf thymus terminal transferase and the chain-terminating base analog [α-^{32}P]cordycepin triphosphate (3′ deoxyribonucleoside triphosphate) (Tu and Cohen 1980). Because these two methods almost always result in the introduction of radiolabel at both termini of the DNA, it is usually necessary to carry out further manipulations to obtain asymmetrically labeled molecules. In some cases, gel electrophoresis can be used to prepare separated strands of the radiolabeled DNA (see, e.g., Hayward 1972). However, this method does not work for every fragment of DNA and typically yields preparations of complementary strands that are only partially pure. More frequently, therefore, the radiolabeled DNA is digested with one or more restriction enzymes, and the double-stranded end-labeled fragments are then purified by conventional agarose or polyacrylamide gel electrophoresis (see Chapter 6). The sole disadvantage of this method is that it requires some prior knowledge of the pattern of cleavage of the DNA by the particular restriction enzyme(s) used.
- By filling recessed 3′ termini with [^{32}P]dNTPs and the Klenow fragment of *E. coli* DNA polymerase I (see Appendix F). Whether this method generates DNAs that are labeled at one or both termini depends on whether both termini are recessed and, if so, on the sequence of the protruding tails. Often, the filling reaction can be set up so that the radiolabeled dNTP is incorporated into only one terminus of the DNA. In this case, there is no need to cleave the radiolabeled DNA with a restriction enzyme or to purify a fragment before proceeding to chemical modification and hydrolysis. Three examples of this type of asymmetric labeling are shown in Figure 13.10.

A useful set of plasmid vectors that facilitate asymmetric labeling of double-stranded fragments of DNA was developed by Volckaert et al. (1984) and subsequently improved Eckert (1987) (see Figure 13.11). These vectors

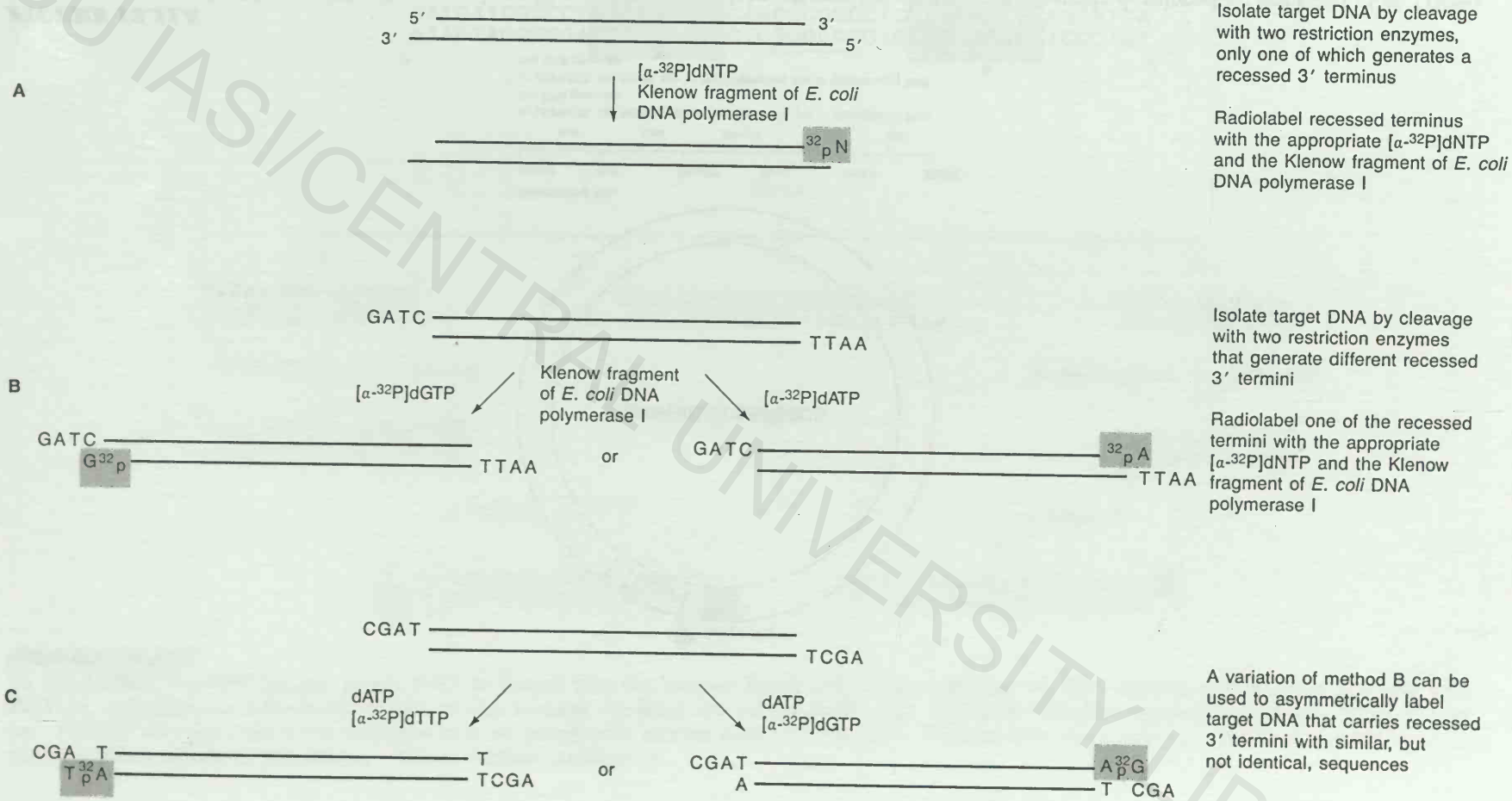

FIGURE 13.10
Examples of methods commonly used to label double-stranded DNA asymmetrically.

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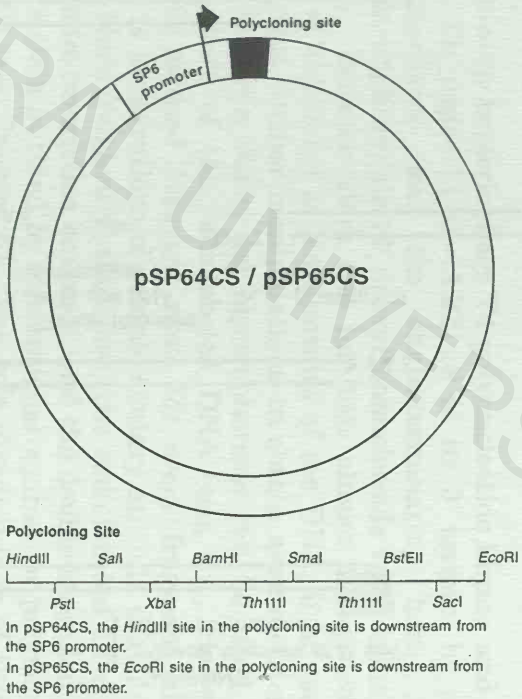

FIGURE 13.11A

Specialized vectors for Maxam-Gilbert sequencing. These vectors are elaborations (Eckert 1987) of plasmids developed by Volckaert et al. (1984) to facilitate chemical sequencing. Their key property is the presence of two of the canonical series of sites recognized by the restriction enzyme *Tth*111I (GACN↓NNGTC). Because these sites differ by a single central base, digestion of pSP64CS/pSP65CS with *Tth*111I generates four termini, each of which can be specifically labeled with a particular [α-^{32}P]dNTP and the Klenow fragment of *E. coli* DNA polymerase I.

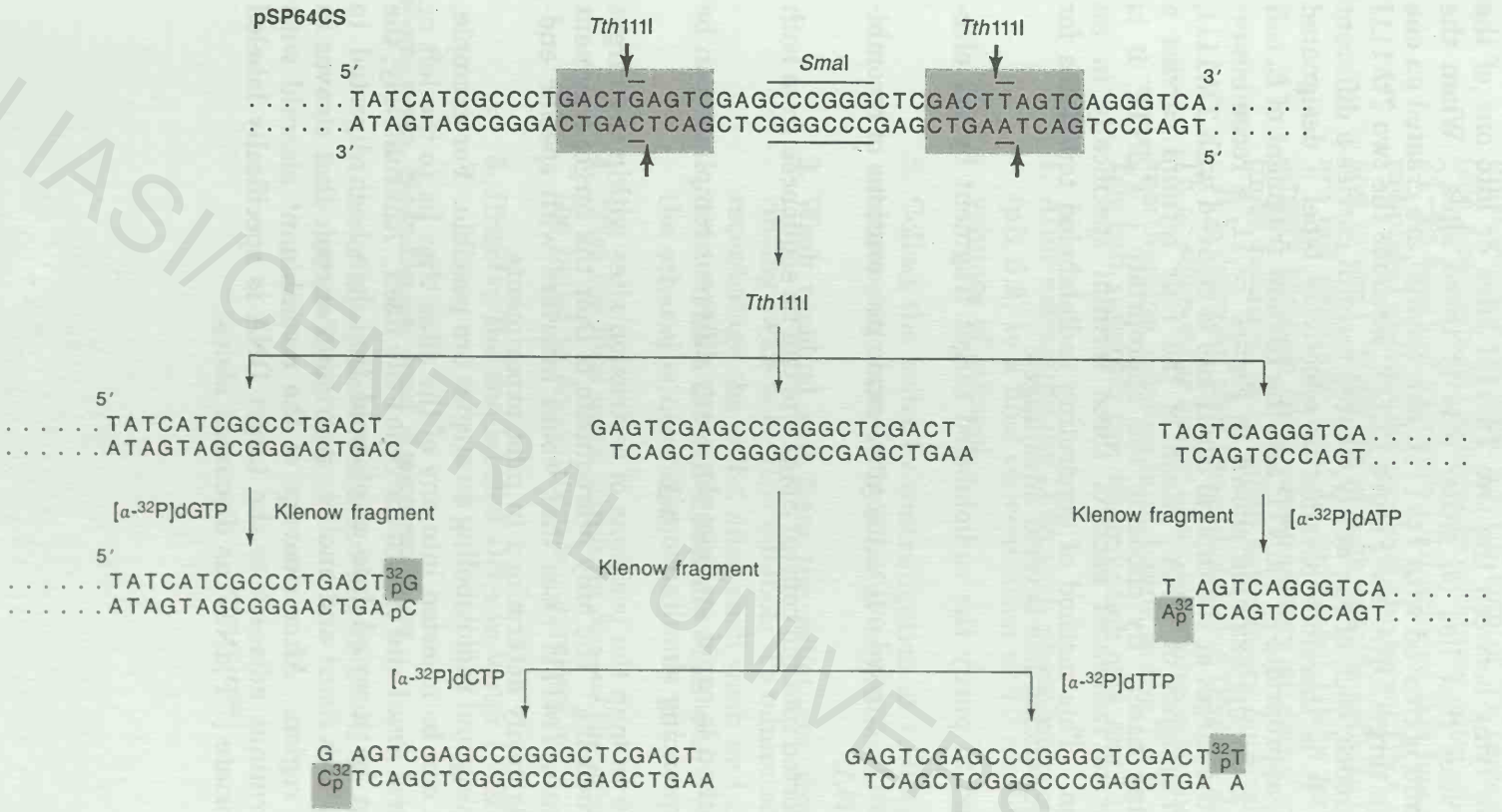

FIGURE 13.11B

To use pSP64CS/pSP65CS, the target DNA is cloned into the central *Sma*I site. After cleavage of the resulting recombinant plasmid with *Tth*111I, radiolabel is introduced at one of the termini flanking the target DNA. The left-hand terminus may be specifically labeled with [α-^{32}P]dCTP and the right-hand terminus may be specifically labeled with [α-^{32}P]dTTP. Because only one terminus is labeled, the DNA can be used for Maxam-Gilbert sequencing without further purification.

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(pSP64CS and pSP65CS) contain a polycloning site with two of the canonical recognition sites (GACN↓NNGTC) for the rarely cutting restriction enzyme *Tth*111I. Recombinant plasmids are created by cloning a target DNA either into the *Sma*I site lying between the two *Tth*111I sites or into one of the other restriction sites that lies on either side of these sites. When the recombinant plasmid is cleaved with *Tth*111I, new termini are created on one or both sides of the target DNA (see Figure 13.11). Because the two *Tth*111I sites differ in sequence, each of the newly created termini carries a different protruding base. It is therefore possible to selectively label a designated terminus with the appropriate [^{32}P]dNTP and the Klenow fragment of *E. coli* DNA polymerase I. In the example shown in Figure 13.11, a recombinant plasmid carrying the target at the central *Sma*I site is cleaved with *Tth*111I, generating two fragments of DNA. Each of the four termini carries a different protruding base. By choosing the appropriate [^{32}P]dNTP, it is therefore possible to radiolabel each of these termini specifically in an end-filling reaction. This method of generating radiolabeled templates for Maxam-Gilbert sequencing has several advantages:

- There is no need to purify the radiolabeled target fragment by gel electrophoresis.
- Radiolabeling can be carried out using small-scale preparations of recombinant plasmid DNA.
- Target DNA inserted into the central *Sma*I site can be sequenced from both ends.
- Because the method is rapid and simple, many different templates can be prepared for sequencing simultaneously.
- There is no need to map the positions of restriction sites within the target DNA before sequencing. The only prerequisite is that the target fragment should not contain *Tth*111I sites that would interfere with specific end-labeling. This is easily determined in pilot experiments.
- A variety of cloning and radiolabeling strategies are possible. For example, the target DNA can be inserted into any of the sites that lie to the left or right of the central *Sma*I site or into the *Sma*I site itself. Alternatively, the target DNA can be inserted into a plasmid that has been digested to completion with both *Sma*I and another restriction enzyme that cleaves in the polycloning region. After cleavage of the recombinant plasmid with *Tth*111I, the terminus adjacent to the target DNA is specifically labeled with the appropriate [^{32}P]dNTP as discussed above.

Preparation of Target DNA for Maxam-Gilbert Sequencing

Whatever the method used for radiolabeling, the end-labeled target DNA must contain at least 5×10^4 cpm of ^{32}P (determined by Cerenkov counting [see Appendix E]) in order to obtain an autoradiograph that can be read after 24–48 hours of exposure. To achieve this level, it is generally necessary to radiolabel at least 0.3 pmole of the target DNA (e.g., 1 μg of a 5-kb fragment) and to use [^{32}P]dNTPs of high specific activity (3000 Ci/mmole). [^{35}S]dNTPs are not generally used to radiolabel DNAs for Maxam-Gilbert sequencing, largely because their lower energy of decay greatly extends the exposure times required to establish satisfactory autoradiographs.

It is *essential* that the radiolabeled DNA be dissolved in water rather than TE or some other buffer. Salt interferes with the reaction between hydrazine and thymine, causing faint bands in the T + C reaction, and buffers such as Tris can suppress the depurination of A and G.

1. After extracting the DNA with phenol:chloroform, add 3 M sodium acetate (pH 5.2) to a final concentration of 0.3 M and precipitate the DNA with 2 volumes of ethanol.

2. Collect the pellet by centrifugation at 12,000*g* for 10 minutes at 4°C, and remove the ethanol by careful aspiration.

3. Wash residual salt from the pellet and from the walls of the tube by rinsing twice with 70% ethanol. To minimize losses of radiolabeled DNA, recentrifuge the pellet after each wash and check the amount of radioactivity in both the pellet and the wash with a hand-held minimonitor before the ethanol is discarded.

4. After the final wash, carefully remove the ethanol by aspiration, and allow the pellet to dry at room temperature.

5. Dissolve the end-labeled DNA in water ($\sim$2500–5000 cpm/μl).

Reagents, Solutions, and Apparatuses

A list of equipment and materials required for Maxam-Gilbert sequencing is provided in Table 13.6.

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TABLE 13.6 Reagents, Solutions, and Apparatuses Required for Maxam-Gilbert Sequencing

1 M Acetic acid	This solution should be freshly diluted from glacial acetic acid (17.4 M).
Dimethyl sulfate (DMS)[a]	99% (Aldrich; Gold label 99%, catalog no. 018 63-009).
10% DMS[a]	This solution is made by mixing 1 volume of dimethyl sulfate with 9 volumes of ethanol.
DMS buffer	50 mM sodium cacodylate (pH 7.0) 1 mM EDTA (pH 8.0)
DMS stop solution	1.5 M sodium acetate (pH 7.0) 1 M β-mercaptoethanol 250 μg/ml yeast tRNA
100% Ethanol	Chill to −20°C.
Hydrazine[b]	95% (Eastman Kodak catalog no. 902). Hydrazine should be stored in small aliquots in tightly capped microfuge tubes at −20°C.
Hydrazine stop solution	0.3 M sodium acetate (pH 7.0) 0.1 mM EDTA (pH 8.0) 100 μg/ml yeast tRNA
5 M NaCl	
NaOH/EDTA solution	1.2 N NaOH 1 mM EDTA
1 M Piperidine in H_2O	This solution should be freshly made by mixing 1 volume of piperidine (10 M; Fisher catalog no. P125-100) with 9 volumes of water in a glass tube. Undiluted piperidine will dissolve most plastics.
1 M Piperidine formate (pH 2.0)	This solution is made by adjusting a 4% solution of formic acid in water to pH 2.0 with 10 M piperidine.
Salmon sperm DNA or calf thymus DNA	Dissolve the DNA at a concentration of 1 mg/ml in water and then sonicate extensively to produce fragments ~500 nucleotides in length (e.g., with six 40-second bursts with a 475-W cup-horn sonicator from Heat Systems Ultrasonics, Inc.). Add NaCl to a final concentration of 0.15 M, and purify the sonicated DNA by three sequential extractions with phenol:chloroform. Concentrate the DNA by precipitation with 2 volumes of ethanol (15 minutes at room temperature) and centrifugation (5000*g* for 15 minutes at 4°C). Wash the precipitate and the walls of the centrifuge tube extensively with 70% ethanol to remove salt. After the final wash, allow the ethanol to evaporate and dissolve the DNA in a small volume of water. Dilute the solution with water until the $OD_{260} = 20$ (i.e., the concentration of DNA is ~1 mg/ml).
Sequencing gel-loading buffer	98% deionized formamide 10 mM EDTA (pH 8.0) 0.025% xylene cyanol FF 0.025% bromophenol blue *Formamide:* Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with

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	Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C. Several companies sell formamide, distilled and packaged under nitrogen, that need not be purified before use (e.g., BRL catalog no. 5515UA).
3 M Sodium acetate (pH 5.2)	
Target DNA	At least 100,000 dpm (25,000–50,000 cpm) of asymmetrically end-labeled DNA, dissolved at a concentration of 2500–5000 cpm/μl in water. It is essential that the DNA solution be free of salt (see page 13.83).
Yeast tRNA stock solution	1 mg/ml tRNA in water (BRL catalog no. 5401SA).
Lyophilizer	To avoid smeary bands, it is essential to remove the last traces of piperidine from the base-specific cleavage reactions. This is best achieved by an efficient rotary vacuum desiccator (e.g., Savant Speed-Vac).
Microfuge tubes	Cleavage reactions are generally carried out in standard microfuge tubes. When sequencing a number of DNAs at the same time, different colored microfuge tubes can be used to facilitate the identification of each base-specific cleavage reaction. Some workers prefer to use siliconized tubes. However, this is unnecessary if care is taken to ensure that all of the radioactive DNA is redissolved after each precipitation, for example, by using a pipette tip to roll a bead of buffer over the entire inner surface of the tube.
Sequencing gels and electrophoresis apparatus	See pages 13.45–13.46.
Temperature control	An ice-water bath is used to chill the reactions to 0°C. Precipitation of DNA is usually carried out at −70°C in a dry-ice/ethanol bath. Heating of the base-cleavage reactions to 90°C is best carried out in an electric heating block containing sand or silicone oil.
The following reagents are needed only for the alternative method of Maxam-Gilbert sequencing.	
Allyl alcohol (2-propen-1-ol)	Fisher catalog no. 1066141.
1-Butanol	
8.8% Formic acid	This solution is made by diluting 1 volume of concentrated formic acid (88%) with 9 volumes of water.
30% NaOH	This solution is made by dissolving 30 g of NaOH in 50 ml of water. (NaOH is dissolved exothermically and it is therefore essential to add the pellets slowly to prevent the solution from boiling.) After the pellets have completely dissolved and the solution has cooled, the volume is adjusted to 100 ml with water.
Potassium permanganate ($KMnO_4$)[c]	2 mg/ml in water. This stock solution may be stored in the dark for up to 1 week. The working solution is made on the day of use by diluting the stock solution 1:100 in water.

[a] **Caution:** Dimethyl sulfate is toxic and a carcinogen and should be handled in a chemical hood. Solutions containing dimethyl sulfate should be discarded in a waste bottle containing 5 N NaOH.

[b] **Caution:** Hydrazine is toxic and explosive in the anhydrous state. It should be handled in a chemical hood. Discard solutions containing hydrazine in a waste bottle containing 3 M ferric chloride.

[c] **Caution:** $KMnO_4$ is an irritant and is explosive. Solutions containing $KMnO_4$ should be handled in a chemical hood.

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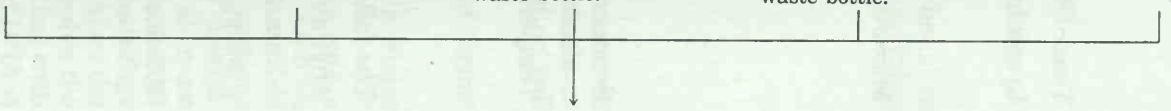
Flowchart of Traditional Method of Maxam-Gilbert Sequencing

G	A+G	C+T	C	A>C
Mix 190 µl DMS buffer 4 µl sonicated DNA 5 µl ^{32}P-labeled DNA	Mix 10 µl H_2O 4 µl sonicated DNA 10 µl ^{32}P-labeled DNA	Mix 10 µl H_2O 4 µl sonicated DNA 10 µl ^{32}P-labeled DNA	Mix 15 µl 5 M NaCl 4 µl sonicated DNA 5 µl ^{32}P-labeled DNA	Mix 100 µl 1.2 N NaOH, 1 mM EDTA 4 µl sonicated DNA 5 µl ^{32}P-labeled DNA
Chill to 0°C.	Chill to 0°C.	Chill to 0°C.	Chill to 0°C.	Close top of tube tightly and secure with plastic tape
Add 5 µl 10% DMS and mix.	Add 4 µl piperidine formate (pH 2.0) and mix.	Add 30 µl hydrazine and mix gently.	Add 30 µl hydrazine and mix gently.	
Incubate 5 minutes at 20°C.	Incubate 15 minutes at 37°C.	Incubate 7 minutes at 20°C.	Incubate 5 minutes at 20°C.	Incubate 7 minutes at 90°C. Chill to 0°C.
Add 50 µl DMS stop solution (at 0°C) and mix.	Add 240 µl hydrazine stop solution (at 0°C) and mix.	Add 200 µl hydrazine stop solution (at 0°C) and mix.	Add 200 µl hydrazine stop solution (at 0°C) and mix.	Add 150 µl 1 M acetic acid, 5 µl tRNA (1 mg/ml) and mix.
Add 750 µl of ethanol (−20°C) and mix.	Add 750 µl of ethanol (−20°C) and mix.	Add 750 µl of ethanol (−20°C) and mix.	Add 750 µl of ethanol (−20°C) and mix.	Add 750 µl of ethanol (−20°C) and mix.
Store for 5 minutes at −70°C.	Store for 5 minutes at −70°C.	Store for 5 minutes at −70°C.	Store for 5 minutes at −70°C.	Store for 5 minutes at −70°C.
Centrifuge 5 minutes at 12,000*g* at 4°C.	Centrifuge 5 minutes at 12,000*g* at 4°C.	Centrifuge 5 minutes at 12,000*g* at 4°C.	Centrifuge 5 minutes at 12,000*g* at 4°C.	Centrifuge 5 minutes at 12,000*g* at 4°C.
Remove the supernatant carefully and dispose of it in the appropriate waste bottle.	Remove the supernatant.	Remove the supernatant carefully and dispose of it in the appropriate waste bottle.	Remove supernatant carefully and dispose of it in the appropriate waste bottle.	Remove the supernatant.



Add 300 μl 0.3 M sodium acetate (pH 5.2) at 0°C and mix by vortexing.

Add 900 μl ethanol (−20°C) and mix.

Store 5 minutes at −70°C.

Centrifuge 5 minutes at 12,000*g* at 4°C.

Remove supernatant carefully.

Add 1 ml ethanol (−20°C) and mix.

Centrifuge 2 minutes at 12,000*g* at 4°C.

Remove supernatant carefully.

Lyophilize pellet of DNA.

Add 100 μl 1 M piperidine. Close tops of tubes tightly and mix.

Incubate 30 minutes at 90°C.

Lyophilize.

Add 20 μl of H_2O. Vortex for 30 seconds.

Centrifuge 10 seconds at room temperature.

Lyophilize.

Add 20 μl of H_2O. Vortex for 30 seconds.

Centrifuge 10 seconds at room temperature.

Lyophilize.

Add 10 μl sequencing gel-loading buffer. Vortex for 20 seconds.

Centrifuge 10 seconds at room temperature.

Close the tops of the tubes tightly. Heat to 90°C for 1 minute.

Load 3 μl of each sample on a lane of a sequencing gel.

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Each of the five base-specific cleavage reactions is described in detail below. Usually, all of these reactions are carried out simultaneously as outlined in the flowchart on pages 13.86–13.87. However, individual base-specific cleavage reactions are occasionally performed to provide DNA markers of known size. In most cases, the cleavage reactions are carried out on one day and the gels are run on the following day. However, with practice, it is possible to complete both cleavage and electrophoresis in a single working day, if a sequencing gel is prepared ahead of time.

When performing Maxam-Gilbert sequencing for the first time or when using a new set of reagents, it is sensible to carry out a set of pilot reactions using a radiolabeled DNA of known sequence. From the results of the pilot reactions, it is possible to adjust the time of each reaction to obtain the maximum amount of information from the radiolabeled DNA of interest.

Caution: Because many of the reagents used in Maxam-Gilbert sequencing are toxic and/or volatile, the degradation reactions should be carried out in a well-ventilated chemical hood.

Cleavage at G residues

1. Mix:

DMS buffer (see Table 13.6)	190 μl
sonicated salmon sperm or calf thymus DNA (1 mg/ml) (see Table 13.6)	4 μl
radiolabeled DNA (2500–5000 cpm/μl)	5 μl

2. Chill the mixture to 0°C.

3. Add 5 μl of 10% DMS (see Table 13.6). Immediately close the cap of the tube, and mix the contents thoroughly by vortexing for 10–15 seconds. Incubate the tube for 5 minutes at 20°C.

   Caution: Dimethyl sulfate is toxic and should be handled in a chemical hood. Solutions containing dimethyl sulfate should be discarded into a waste bottle containing 5 N NaOH.

   The original Maxam-Gilbert protocol called for addition of 1 μl of undiluted DMS. However, using a freshly diluted solution of DMS minimizes variation in the extent of G-specific cleavage due to errors in pipetting small volumes of the reagent.

4. Add 50 μl of "DMS stop solution" (see Table 13.6) chilled to 0°C. Mix the solution by vortexing, and then add 750 μl of ethanol chilled to −20°C.

5. Mix the solution by vortexing, and then store the tube for 5 minutes at −70°C in a dry-ice/ethanol bath.

6. Recover the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

7. Using a 1-ml disposable pipette tip, transfer the supernatant to the DMS waste bottle. Use a yellow tip to remove any residual solution from the walls of the tube.

8. To the pellet of DNA, add 300 μl of 0.3 M sodium acetate (pH 5.2) chilled to 0°C.

9. Close the top of the tube, and vortex the solution. Add 900 μl of ethanol chilled to −20°C. Close the tube, mix the solution by vortexing, and then store the tube for 5 minutes at −70°C.

10. Recover the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

11. Remove the supernatant, and add 1 ml of ethanol chilled to −20°C. Close the tube, and mix the solution by vortexing.

12. Recover the DNA by centrifugation at 12,000*g* for 2 minutes at 4°C in a microfuge.

13. Remove as much of the supernatant as possible, and then dry the pellet of DNA in a lyophilizer for 5–10 minutes.

14. Treat the DNA with piperidine as described on page 13.94.

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Cleavage at Purine Residues (A + G)

1. Mix:

H_2O	10 μl
sonicated salmon sperm or calf thymus DNA (1 mg/ml) (see Table 13.6)	4 μl
end-labeled DNA (2500–5000 cpm/μl)	10 μl

2. Chill the mixture to 0°C.

3. Add 4 μl of 1 M piperidine formate (pH 2.0) (see Table 13.6). Close the cap of the tube. Mix by vortexing, and incubate the tube for 15 minutes at 37°C.

4. Add 240 μl of "hydrazine stop solution" (see Table 13.6) chilled to 0°C, mix the solution by vortexing, and then add 750 μl of ethanol chilled to −20°C. Again mix by vortexing, and store the tube for 5 minutes at −70°C in a dry-ice/ethanol bath.

5. Recover the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

6. Remove the supernatant, and then process the precipitated DNA as described in steps 8–14 on page 13.89.

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Cleavage at Pyrimidine Residues (C + T)

1. Mix:

H_2O	10 μl
sonicated salmon sperm or calf thymus DNA (1 mg/ml) (see Table 13.6)	4 μl
end-labeled DNA (2500–5000 cpm/μl)	10 μl

2. Chill the mixture to 0°C.

3. Add 30 μl of 95% hydrazine, close the top of the tube, and mix the solution by gently tapping the tube with a finger.

   Caution: Hydrazine is toxic and explosive in the anhydrous state. It should be handled in a chemical hood. Solutions containing hydrazine should be discarded in a waste bottle containing 3 M ferric chloride.

4. Incubate the mixture for 7 minutes at 20°C.

5. Add 200 μl of "hydrazine stop solution" (see Table 13.6) chilled to 0°C. Mix the solution by vortexing, and then add 750 μl of ethanol chilled to −20°C.

6. Mix the solution by vortexing, and then store the tube for 5 minutes at −70°C in a dry-ice/ethanol bath.

7. Recover the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

8. Using a 1-ml disposable pipette tip, transfer the supernatant to the hydrazine waste bottle. Use a yellow tip to remove any residual solution from the walls of the tube.

9. Process the precipitated DNA as described in steps 8–14 on page 13.89.

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Cleavage at C Residues

1. Mix:

5 M NaCl	15 μl
sonicated salmon sperm or calf thymus DNA (1 mg/ml) (see Table 13.6)	4 μl
end-labeled DNA (2500–5000 cpm/μl)	5 μl

2. Chill the mixture to 0°C.

3. Add 30 μl of 95% hydrazine, close the top of the tube, and mix the solution by gently tapping the tube with a finger.

   Caution: Hydrazine is toxic and explosive in the anhydrous state. It should be handled in a chemical hood. Solutions containing hydrazine should be discarded in a waste bottle containing 3 M ferric chloride.

4. Incubate the mixture for 5 minutes at 20°C.

5. Add 200 μl of "hydrazine stop solution" (see Table 13.6) chilled to 0°C. Mix the solution by vortexing, and then add 750 μl of ethanol chilled to −20°C.

6. Mix the solution by vortexing, and then store the tube for 5 minutes at −70°C in a dry-ice/ethanol bath.

7. Recover the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

8. Using a 1-ml disposable pipette tip, transfer the supernatant to the hydrazine waste bottle. Use a yellow tip to remove any drops of solution remaining on the walls of the tube.

9. Process the precipitated DNA as described in steps 8–14 on page 13.89.

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Cleavage at A and C Residues (A > C)

1. Mix:

NaOH/EDTA solution (see Table 13.6)	100 μl
sonicated salmon sperm or calf thymus DNA (1 mg/ml) (see Table 13.6)	4 μl
end-labeled DNA (2500–5000 cpm/μl)	5 μl

2. Close the top of the tube securely. If necessary, centrifuge the tube briefly to deposit all of the fluid in the bottom.

3. Incubate the tube for 7 minutes at 90°C.

   To prevent the top of the tube from popping open during heating, either place a heavy weight on it or seal the top with plastic tape.

4. Chill the tube to 0°C, and then add 150 μl of 1 M acetic acid, followed by 5 μl of yeast tRNA stock solution (1 mg/ml) (see Table 13.6). Mix by vortexing.

5. Add 750 μl of ethanol chilled to −20°C. Mix the solution by vortexing, and then store the tube for 5 minutes at −70°C in a dry-ice/ethanol bath.

6. Recover the DNA by centrifugation at 12,000*g* for 5 minutes at 4°C in a microfuge.

7. Remove the supernatant, and then process the precipitated DNA as described in steps 8–14 on page 13.89.

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Treatment of Samples with Piperidine

Piperidine is used to cleave the sugar-phosphate chain of DNA at the sites of chemical modification.

1. Resuspend each of the five lyophilized DNA samples obtained by the procedures described on pages 13.88–13.93 in 100 μl of 1 M piperidine.

2. Close the tops of the tubes securely. If necessary, centrifuge the tubes briefly to deposit all of the fluid on the bottom.

3. Incubate the tubes for 30 minutes at 90°C.

   To prevent the tops of the tubes from popping open during heating, either place a heavy weight on the tubes or seal the tops with plastic tape.

4. After the tubes have cooled to room temperature, open them and evaporate all of the samples to dryness in a rotary vacuum lyophilizer (e.g., Savant SpeedVac).

   This step usually takes 1–4 hours depending on the efficiency of the lyophilizer.

5. Remove the tubes from the lyophilizer, and add 20 μl of H_2O to each tube. Close the caps of the tubes, and vortex the tubes for 30 seconds to redissolve the DNA. Centrifuge the tubes briefly to deposit all of the fluid on the bottom.

   Use a hand-held minimonitor to check that all of the radiolabeled DNA has been washed from the walls of the tubes by the water and is redissolved in the fluid.

6. Open the tubes, and evaporate all of the samples to dryness in a rotary vacuum lyophilizer.

   This step usually takes 15–30 minutes, depending on the efficiency of the lyophilizer.

7. Repeat steps 5 and 6.

   To ensure that all of the piperidine has been removed, some workers prefer to redissolve the DNA once more in 10 μl of H_2O and to carry out an additional cycle of lyophilization. However, this is necessary only if the samples have an "oily" look or if abnormally long periods of lyophilization have been required to reduce them to dryness.

8. Add to each tube 10 μl of sequencing gel-loading buffer (see Table 13.6). Vortex the tubes to redissolve the DNA. Centrifuge the tubes briefly to deposit all of the fluid on the bottom.

9. Close the tops of the tubes, and heat the tubes for 1 minute at 90°C to denature the DNA. Immediately load 3 μl of each of the reaction mixtures onto separate lanes of a sequencing gel, prepared as described on pages 13.45–13.53.

Note

A flowchart of Maxam-Gilbert reactions is given on pages 13.86–13.87.

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ALTERNATIVE METHODS OF MAXAM-GILBERT SEQUENCING

There are a number of alternative base-specific cleavage reactions that are occasionally used to resolve doubtful regions of sequence or to confirm a known sequence of DNA. However, the results are generally not as clean as those obtained from the traditional set of five base-cleavage reactions. A flowchart for rapid Maxam-Gilbert sequencing is given on pages 13.96–13.97.

TROUBLESHOOTING GUIDE FOR MAXAM-GILBERT SEQUENCING

Reading Sequencing Gels

It is more difficult to determine the sequence of DNA from a chemical-degradation sequencing gel than from a Sanger dideoxy-mediated chain-termination sequencing gel. Because the chemical cleavage reactions are not all absolutely base-specific, it is necessary to deduce the position of T residues by subtracting bands appearing in the C track from those in the C + T track. Similarly, the position of A residues is deduced by subtracting bands in the G track from those in the A + G track. The presence of an A residue is confirmed if a strong band is present in the A > C track.

Because Maxam-Gilbert sequencing is invariably carried out with ^{32}P-labeled DNA, the bands are inevitably fuzzier and broader than those obtained from DNA sequenced by the Sanger method, which uses [^{35}S]dNTPs. This lack of resolution limits the amount of reliable sequence that can be obtained from a single gel to approximately 200–250 nucleotides.

Problems Commonly Encountered

If difficulties arise with chemical sequencing, the best course of action is to read the article by Maxam and Gilbert (1980) which explains in detail how to diagnose and correct problems. Table 13.7 is reproduced, with permission, from this article.

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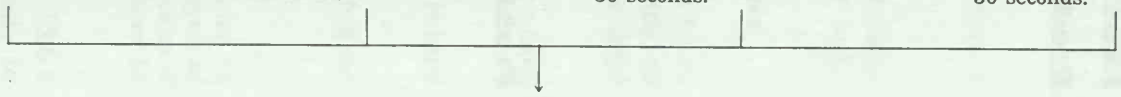
Flowchart of Rapid Method of Maxam-Gilbert Sequencing

A+G	A>C	C+T (C>T)	T
Mix 4 μl sonicated DNA 10 μl ^{32}P-labeled DNA	Mix 4 μl sonicated DNA 5 μl ^{32}P-labeled DNA	Mix 4 μl sonicated DNA 10 μl ^{32}P-labeled DNA	Mix 4 μl sonicated DNA 5 μl ^{32}P-labeled DNA
Add 3 μl 8.8% formic acid.	Add 1 μl 30% NaOH.	Add 15 μl hydrazine.	
Incubate 7 minutes at 37°C.	Incubate 6 minutes at 90°C.	Incubate 4 minutes at 20°C.	Incubate 2 minutes at 90°C.
Chill to 0°C.	Chill to 0°C.	Chill to 0°C.	Chill to 0°C.
Add 150 μl 1 M piperidine (at 0°C).	Add 150 μl 1 M piperidine (at 0°C).	Add 1 ml 1-butanol. Vortex for 30 seconds.	Add 20 μl $KMnO_4$ (20 μg/ml).
Store on ice until all reactions are ready for incubation.	Store on ice until all reactions are ready for incubation.	Centrifuge at 12,000*g* for 2 minutes at 4°C.	Incubate for 8 minutes at 20°C.
		Remove *all* supernatant.	Add 10 μl allyl alcohol. Vortex for 30 seconds.
		Add 150 μl H_2O. Add 1.2 ml 1-butanol. Vortex for 30 seconds.	Lyophilize.
		Centrifuge at 12,000*g* for 2 minutes at 4°C.	Add 150 μl 1 M piperidine.
		Remove all supernatant.	Store on ice until all reactions are ready for incubation.
		Lyophilize.	
		Add 150 μl 1 M piperdine.	
		Store on ice until all reactions are ready for incubation.	
Incubate 30 minutes at 90°C.	Incubate 30 minutes at 90°C.	Incubate 30 minutes at 90°C.	Incubate 30 minutes at 90°C.
	Add 150 μl ethanol (70%).		
Add 1.2 ml 1-butanol. Vortex for 30 seconds.	Add 1.2 ml 1-butanol. Vortex for 30 seconds.	Add 1.2 ml 1-butanol. Vortex for 30 seconds.	Add 1.2 ml 1-butanol. Vortex for 30 seconds.



Centrifuge 2 minutes at 12,000*g* at 4°C.

Remove supernatant *completely*.

Add 150 μl 1% SDS, 1–2 ml 1-butanol. Vortex for 30 seconds.

Centrifuge 2 minutes at 12,000*g* at room temperature.

Remove supernatant *completely*.

Lyophilize for 20 minutes.

Add 10 μl sequencing gel-loading buffer. Vortex for 20 seconds.

Centrifuge 10 seconds at 12,000*g* at room temperature.

Close the tops of the tubes tightly. Heat to 90°C for 1 minute.

Load 3 μl of each sample on a lane of a sequencing gel.

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TABLE 13.7 Diagnosis and Correction of Aberrations in Sequencing Band Patterns

Problem	Probable causes	Suggested solutions
1. Any or all of the following: a. Smearing of pyrimidine cleavage products b. Loss of electrophoresis dye color in C and/or C + T samples c. Bands in C and/or C + T ladders at positions where purines follow pyrimidines in the sequence d. Large, water-insoluble pellets after the first ethanol precipitation e. Poor suppression of Ts in the C ladder	Fragments of DNA broken at pyrimidines contain one or more internal thymines and/or cytosines that reacted with hydrazine but did not then cleave with piperidine. Each of these legitimate cleavage products blurs because its charge and/or mass is heterogeneous, due to the varying number and nature of internal hydrazine reaction products. Causes include an inadequate piperidine reaction and secondary reactions with residual hydrazine during the 90°C strand-scission and heat denaturation steps. Hydrazine can be carried through the procedure when ethanol precipitation and/or lyophilization fails to remove it. An insoluble complex of hydrazine, magnesium, and possibly other ions, which appears as a larger pellet, and poor vacuum during the lyophilization steps are common causes of this problem.	If magnesium acetate is still used in the hydrazine stop solution as originally suggested, delete it. If piperidine reactions are done in microfuge tubes, be sure to use at least 100 μl and to seal the cap tightly (otherwise, use flame-sealed capillaries). After the piperidine reaction, employ a strong vacuum with rewetting of the residue to ensure removal of any residual hydrazine during lyophilization.
	Hydrazine has undergone oxidation.	Replace the hydrazine with a fresh aliquot or new stock bottle.
2. Ts are weak in the C + T ladder.	Residual salt from ethanol precipitation of end-labeled DNA is carried over into the sequencing reactions and partially suppresses the reaction of hydrazine with thymine in the C + T sample.	As a final purification step, dissolve end-labeled DNA in 25 μl of H_2O and 1 ml of ethanol, mix, chill, centrifuge, and dry the pellet. Then dissolve it in water and distribute into sequencing reactions.
3. Bands are present in C and/or C + T ladders at *all* guanine positions, not just those where guanine follows a pyrimidine in the sequence as in 1c above or 4 below.	A reaction between hydrazine and guanine at lower pH (sodium acetate), after which piperidine breaks the DNA at guanines.	Keep the samples at 4°C or below during the ethanol precipitation steps. Use chilled (0–4°C) hydrazine stop solution, sodium acetate, and ethanol.
4. Bands in C and/or C + T ladders at positions where purines follow pyrimidines in the sequence, but not at every purine in a run as in 3 above. See also problem 1c above.	Residual sodium acetate from the second ethanol precipitation buffers piperidine, leading to incomplete phosphate elimination reactions, which require a high pH. The 3′ termini of 5′-labeled fragments then retain deoxyribosyl residues, have one less negative charge, and move one position slower in the gel.	Rinse the last pellet well with 95% ethanol or dissolve it in 25 μl of H_2O; add 1 ml of 95% ethanol, mix, chill, and collect the reprecipitated DNA by centrifugation.
	Some labeled DNA is not dissolved in the piperidine and therefore does not react with it at 90°C.	After adding piperidine to a tube containing dry DNA, close the cap and bat the tube with fingers to splash the liquid up the walls; then collect it at the bottom with a quick spin.

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5. All DNA bands and both electrophoresis dyes are retarded in some ladders.	Effect of residual piperidine on gel electrophoresis.	Wet the DNA residue, freeze, and lyophilize with a strong vacuum as suggested in chemical cleavage protocols.
6. Bands in A > G ladders produced by the older R2 reaction[a] are not sharp.	Overmethylation. Fragments cleaved at adenines carry too many ring-opened 7-methylguanines and are heterogeneous in charge.	Employ the same dimethyl sulfate reaction conditions used for reaction R5[a] guanine cleavage. (Weak A > G bands can be strengthened by mixing more often during the acid release, slightly increasing the acid concentration, or introducing extra labeled DNA into samples destined for A > G cleavage.)
7. All ladders exhibit uniform chemical cleavage of desired base specificity, but the bands are not sharp enough to permit reading very far into the sequence.	Sample not evenly distributed in sample well.	Layer samples with a pointed glass capillary, using a slow, side-to-side, sweeping motion.
	Diffusion or secondary structural effects during electrophoresis.	Turn the power up until the gel runs at 50°C.
	Parallax or scatter during autoradiography.	Use a slab gel no more than 1.5 mm thick, preferably 0.5 mm, and compress the X-ray film against it with lead bricks during autoradiography.
8. Sudden loss of expected bands, band spacing, or band order in all four ladders in the middle of a sequencing pattern.	Nested end-labeled cleavage products become long enough to fold into stable hairpins and move anomalously in the gel (the so-called compression effect).	Be sure that the DNA is thoroughly denatured when loaded on the sequencing gel. Prerun and run the gel at a voltage that generates as much heat as the glass plates will tolerate (>50°C).
9. Loss of just one band in both pyrimidine ladders in the middle of a sequencing pattern.	Presence of 5-methylcytosine, which reacts so slowly with hydrazine (relative to unmodified cytosine) that the DNA is not cleaved at that position.	Look for guanine at the corresponding position in the sequence of the complementary strand.
10. Extraneous bands in all ladders throughout a sequencing pattern.	Contaminating end-labeled fragment.	Strive for better DNA resolution on the fragment-division or strand-separation gel, or use a different restriction enzyme for secondary cleavage.
	Terminal heterogeneity generated before or during end-labeling by a contaminating nuclease.	Purify restriction enzyme, phosphatase, kinase, or terminal transferase.
	Unavoidable terminal heterogeneity originating in vivo or as a consequence of DNAase I or nuclease S1 treatment in vitro.	After labeling ragged ends, cleave with restriction enzymes that will give end segments less than 100 bp long, denature, and fractionate on a 1.5-mm 8% sequencing gel. Strands differing in length by one or more nucleotides will resolve and be extracted for sequence analysis.

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Problem	Probable causes	Suggested solutions
11. An extraneous band across all four ladders at one position in a sequencing pattern.	Some double-stranded DNA molecules are nicked by a restriction enzyme at a sequence that closely resembles the enzyme's true recognition site or by a sequence-specific contaminating enzyme. After end-labeling, a double-stranded fragment isolated on a nondenaturing gel retains the hidden break, which is then revealed on the denaturing sequencing gel. This can be confirmed by electrophoresis of some unreacted end-labeled DNA in parallel with the sequencing samples. Unique endogenous nicks and alkali-labile nucleotides are unlikely causes, but they are possible in rare cases and subject to similar tests.	Attempt to derive the sequence surrounding the break on both strands, and compare it with the recognition sequences of all restriction enzymes used to generate that DNA fragment. In making this comparison, remember that bands in the sequencing pattern arise from fragments that lack the nucleotide with which they are identified, whereas enzymatic cleavage products retain all nucleotides. If $\frac{3}{4}$, $\frac{4}{5}$, or $\frac{5}{6}$ of the bases are homologous, tentatively assume nicking at the variant site. Then avoid excesses of that restriction enzyme in subsequent DNA digestions, ideally using just enough pure enzyme to cleave every legitimate site. If the problem persists, avoid that enzyme altogether and use another cleavage route or strand separation.
12. One or a few bands in two ladders produced by cleavage reactions that do not normally share base specificity. Cleavage is consistent with base specificity everywhere else in the sequencing pattern.	Sequence heterogeneity in the DNA at that position. Compression.	Confirm by getting the complementary effect on the other strand. See 8 above.
13. Difficulty in identifying the band corresponding to chemical cleavage of the ultimate 5′ nucleotide (^{32}P-labeled with polynucleotide kinase and [γ-^{32}P]ATP).	[^{32}P]Orthophosphate is the product of base-specific chemical cleavage of the ^{32}P-labeled 5′ terminal nucleotide, and this migrates so rapidly that it is usually run off the gel and missed altogether. A diffuse hydrazinolysis artifact product that sometimes appears between the first and third bands further confuses the interpretation.	Put some [^{32}P]phosphate in formamide–buffer–dyes solution, and run as a marker adjacent to products of the four base-specific reactions on a 20% sequencing gel. Prerun the gel, load the five samples, electrophorese until the bromophenol blue marker has proceeded one third of the way down the gel, and autoradiograph. A band in one or two of the four usual lanes running even with the phosphate in the fifth indicates which base-specific reaction(s) cleaved the 5′ terminal nucleotide.
14. Difficulty in identifying the band corresponding to chemical cleavage of the ultimate 3′ nucleotide (^{32}P-labeled and incorporated by terminal transferase or DNA polymerase).	With DNA 3′ end-labeled by addition of more than one ^{32}P-labeled nucleotide, chemical attack at the *ultimate,* added 3′ ribonucleotide releases [^{32}P]phosphate, which is usually run off the sequencing gel and missed altogether. Furthermore, be aware that none of the four recommended reactions (G, A + G, T + C, C) will cleave riboadenylic acid, since ribonucleotides do not depurinate readily. The three other ribonucleotides should cleave like deoxynucleotides in these reactions.	Put some [^{32}P]phosphate in formamide–buffer–dyes solution, and run adjacent to products of the four base-specific reactions on a 20% sequencing gel as described under the solution to 13 above. This marker will indicate the product of a reaction with the *ultimate* 3′ base.

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	With DNA 3′ end-labeled with DNA polymerase, chemical attack at the *penultimate* deoxynucleotide releases the ^{32}P-labeled ultimate 3′ nucleotide intact.	Digest some of the end-labeled DNA with DNAase and venom phosphodiesterase, lyophilize, dissolve in formamide–buffer–dyes, and run adjacent to products of the four base-specific reactions on a 20% sequencing gel as described under the solution to 13. This marker will indicate the product of a reaction with the *penultimate* 3′ base.
15. More than one strong band appears at the position of uncleaved DNA at the top of all sequence ladders.	One is single-stranded DNA and the other double-stranded DNA.	Increase dimethyl sulfate or hydrazine reaction times, employ stronger denaturation before loading samples on the gel, or both.
	Variant or incomplete restriction enzyme cleavage.	See 11 above.
	Contaminating end-labeled fragment.	See 10 above.
	Fragment length heterogeneity.	See 10 above.

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[a] For descriptions of R2 and R5 reactions, see Maxam and Gilbert (1980).

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14
In Vitro Amplification of DNA by the Polymerase Chain Reaction

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The polymerase chain reaction (PCR) is used to amplify a segment of DNA that lies between two regions of known sequence. As shown in Figure 14.1, two oligonucleotides are used as primers for a series of synthetic reactions that are catalyzed by a DNA polymerase. These oligonucleotides typically have different sequences and are complementary to sequences that (1) lie on opposite strands of the template DNA and (2) flank the segment of DNA that is to be amplified. The template DNA is first denatured by heating in the presence of a large molar excess of each of the two oligonucleotides and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerase. The cycle of denaturation, annealing, and DNA synthesis is then repeated many times. Because the products of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of the desired DNA product.

The major product of this exponential reaction is a segment of double-stranded DNA whose termini are defined by the 5′ termini of the oligonucleotide primers and whose length is defined by the distance between the primers. In addition, as shown in Figure 14.1, longer DNA molecules are generated during the reaction. For example, the products of a successful first round of amplification are heterogeneously sized DNA molecules, whose lengths may exceed the distance between the binding sites of the two primers. In the second round, these molecules generate DNAs of defined length that will accumulate in an exponential fashion in later rounds of amplification and will form the dominant products of the reaction. Although longer molecules continue to be produced from the original template DNAs in every round, they accumulate only at a linear rate and therefore do not contribute significantly to the final mass of target sequences.

The original protocols for polymerase chain reactions (Saiki et al. 1985; Mullis et al. 1986; Mullis and Faloona 1987) used the Klenow fragment of *E. coli* DNA polymerase I to catalyze the extension of the annealed oligonucleotide primers. Because this enzyme is inactivated at the temperatures that are required to denature DNA, each round of synthesis required the addition of a fresh aliquot of enzyme. Although these reactions generally worked well for the amplification of small segments of DNA (<200 bp), the results with larger templates were disappointing. Frequently, the yield was poor, and the products were often heterogeneous in size (Scharf et al. 1986), presumably because "mispriming" occurred at the temperatures used for catalytic extension of the oligonucleotides. In addition, some of the products were "shuffled" and consisted of mosaics of different alleles of the target sequences (Saiki et al. 1988b).

These problems were solved with the introduction (Saiki et al. 1988b) of a thermostable DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus* (*Taq* DNA polymerase; Chien et al. 1976). This enzyme, which can survive extended incubation at 95°C, is not inactivated by the heat denaturation step and does not need to be replaced at every round of the amplification cycle. In addition, because annealing and extension of oligonucleotides can be carried out at elevated temperatures, mispriming is greatly reduced. This results in substantial improvements in the specificity and yield of the amplification reaction and in the size of the amplified product. For

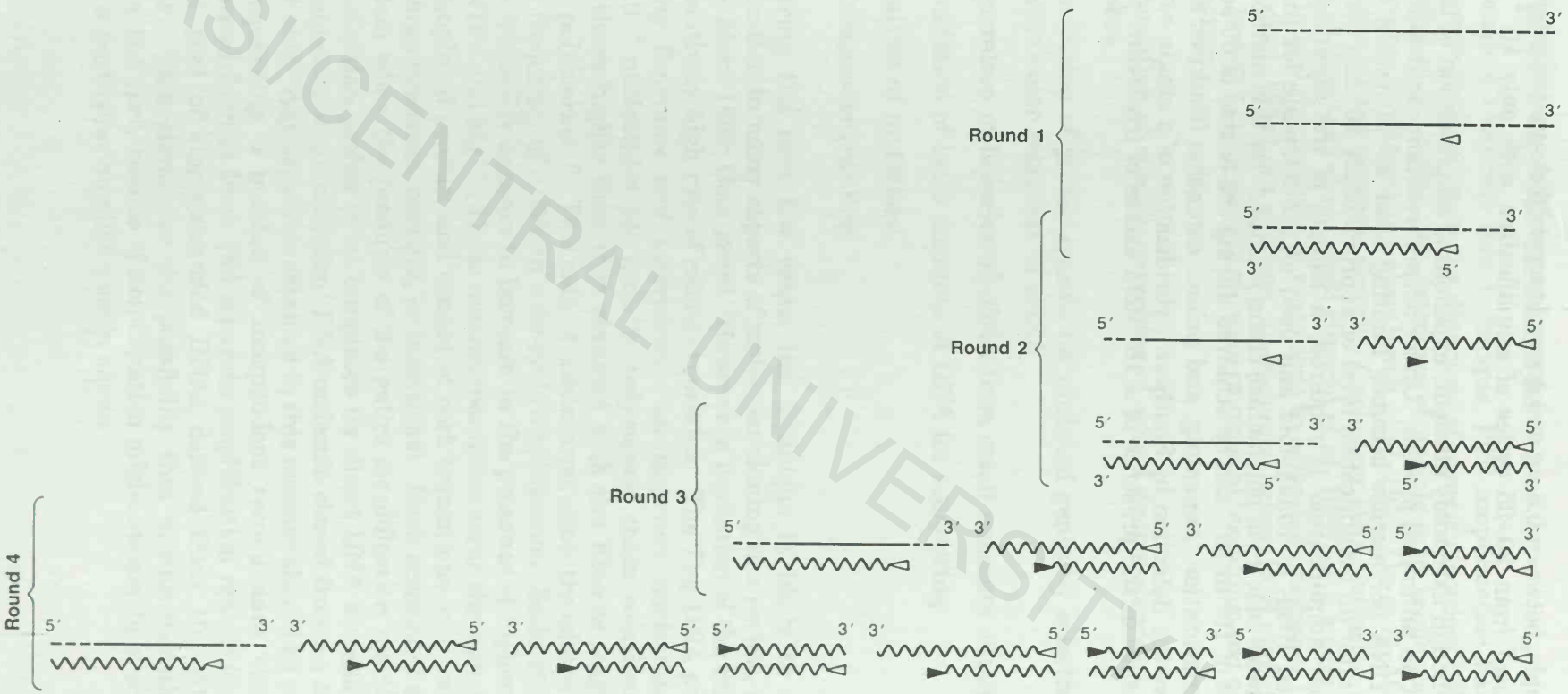

FIGURE 14.1

The diagram shows the steps involved in the first few rounds of a polymerase chain reaction. The original template (top line) is single-stranded DNA and the leftward and rightward oligonucleotide primers are shown as ◁ and ▶, respectively. The products of the first few rounds of the amplification reaction are heterogeneous in size. However, the tract of DNA lying between the two primers is preferentially amplified and quickly becomes the dominant product of the amplification reaction. If the original template is double-stranded, an equivalent set of reactions will take place using the complementary strand as template.

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example, between 0.5 μg and 1 μg of target DNAs up to 2 kb in length can be obtained from 30–35 cycles of amplification with only 10^{-6} μg of starting DNA.

Although extremely efficient, exponential amplification of target sequences is not an unlimited process. Under normal reaction conditions, the amount of *Taq* DNA polymerase becomes limiting after 25–30 cycles of amplification (i.e., after an amplification level of approximately 10^6 has been attained). If further amplification is required, a sample of the amplified DNA can be diluted 1000- to 10,000-fold and used as the template for further rounds of synthesis in a fresh polymerase chain reaction. By this method, amplification levels of 10^9 to 10^{10} can be achieved during the course of 60 sequential cycles of denaturation, annealing, and primer extension (Saiki et al. 1988b). This allows the detection by Southern hybridization of a single copy of the target sequence in the presence of a 10^{13}-fold excess of irrelevant DNA.

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APPLICATIONS OF PCR AMPLIFICATION

Although a relatively new technique, PCR amplification has already found extensive application in the diagnosis of genetic disorders (Wong et al. 1987; Engelke et al. 1988), the detection of nucleic acid sequences of pathogenic organisms in clinical samples (Kwok et al. 1987; Ou et al. 1988), the genetic identification of forensic samples (including DNA extracted from individual hairs [Almoguera et al. 1988; Higuchi et al. 1988] or a single sperm [Li et al. 1988]), and the analysis of mutations in activated oncogenes (Bos et al. 1987; Farr et al. 1988). In addition, as discussed below, PCR amplification is being used to carry out a variety of tasks in molecular cloning and analysis of DNA, including:

- Generation of specific sequences of cloned double-stranded DNA for use as probes
- Generation of probes specific for uncloned genes by selective amplification of particular segments of cDNA
- Generation of libraries of cDNA from small amounts of mRNA
- Generation of large amounts of DNA for sequencing
- Analysis of mutations
- Chromosome crawling

During the next few years, the technique is likely to find increasing application in many aspects of molecular cloning (for review, see Erlich et al. 1988; Marx 1988; Oste 1988). However, a limitation of the current method is its relatively high rate of misincorporation. The *Taq* DNA polymerase lacks editing functions and incorporates an incorrect nucleotide at a rate of 2×10^{-4} nucleotides per cycle in polymerase chain reactions, a rate about four times higher than that obtained with the Klenow fragment of *E. coli* DNA polymerase I. This rate of misincorporation translates into an overall error frequency of 0.25% in a 30-cycle amplification (Saiki et al. 1988b). The error frequency appears to increase in the presence of higher concentrations of dNTPs and Mg^{++}. These misincorporations occur throughout the length of the amplified product and consist of both transitions and transversions (but not large deletions, mosaics, or insertions). Such occasional errors are not a problem when the products of the entire amplification reaction are used as hybridization probes or as templates for direct DNA sequencing. However, the sequence of an *individual* DNA molecule cloned from an amplified pool is unreliable. Any sequence obtained in this manner should be confirmed either by sequencing a number of independent recombinant clones, preferably generated from at least two separate amplification reactions, or by sequencing a pool of single-stranded DNAs derived from 100–200 recombinant clones. This eliminates the possibility that misincorporation(s) occurring during the early rounds of amplification might appear in every clone derived from a particular amplification reaction.

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Generation of Specific Sequences of Cloned Double-stranded DNA for Use as Probes

The polymerase chain reaction can be used, for example, to amplify cDNAs carried in bacteriophage λ vectors by using oligonucleotide primers that anneal to the flanking vector sequences (Saiki et al. 1988b). In some cases, this can eliminate the need for subcloning into plasmid or bacteriophage M13 vectors or for the isolation of specific segments of DNA prior to subcloning. Because the termini of the amplified segment are defined by the sequences of the priming oligonucleotides, the polymerase chain reaction can be used to eliminate unwanted sequences flanking the target DNA. It is therefore possible to generate a series of precisely defined deletion mutants in a set of polymerase chain reactions that are primed by a progressive series of nested oligonucleotides. In addition, useful sequences (e.g., restriction sites) can be inserted at the termini of the amplified fragment(s) by incorporating additional nucleotides at the 5′ termini of the priming oligonucleotides. The polymerase chain reaction therefore provides a very versatile tool to increase the speed and precision of many of the methods used to manipulate DNA in molecular cloning.

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Generation of Probes Specific for Uncloned Genes by Selective Amplification of Particular Segments of cDNA

Several methods have been devised to use the polymerase chain reaction to generate probes for genes or cDNAs that have not yet been cloned. For example, when a limited amount of amino acid sequence is available from the protein of interest, degenerate pools of oligonucleotides can be used to amplify target sequences contained in a pool of first-strand cDNA. One pool consists of oligonucleotides that encompass all possible ways of coding for a particular tract of amino acids in a protein. The other consists of sequences complementary to all possible sequences coding for a second tract of amino acids some distance away in the protein. If desired, one or both pools of oligonucleotides can contain inosine at positions of high degeneracy. mRNA extracted from cells that synthesize the protein is used as a template for synthesis of the first strand of cDNA catalyzed by reverse transcriptase. Both pools of oligonucleotides are then used to prime a polymerase chain reaction that uses this first strand of cDNA as a template. The amplified products of the reaction can then be cloned in a suitable vector, sequenced, and used as probes in Southern or northern hybridizations or for screening cDNA and genomic DNA libraries (Lee et al. 1988).

The obvious advantage of this method (MOPAC, *m*ixed *o*ligonucleotide *p*rimed *a*mplification of *c*DNA) is that it rapidly yields a probe of unique sequence. In most cases, this probe will be long enough to allow hybridization to be carried out under conventional conditions rather than under conditions established empirically for pools of redundant oligonucleotides (see Chapter 11). However, the method has another, potentially greater advantage, namely, the selection for priming by the "correct" oligonucleotides in each of the two pools. Oligonucleotides that are not exactly complementary to the cDNA encoding the protein of interest will be less effective as primers over the course of many rounds of amplification. Lee et al. (1988) used polymerase chain reactions catalyzed by the Klenow fragment of *E. coli* DNA polymerase I and analyzed the sequences of seven independent clones that contained an amplified DNA fragment of porcine urate oxidase cDNA 112 bp in length. Although the sequences of all seven clones of amplified DNA corresponded exactly to the sequence of the authentic porcine urate oxidase gene, each of them carried a different combination of upstream and downstream primers. Thus, the polymerase chain reaction catalyzed by the Klenow fragment of *E. coli* DNA polymerase I can generate amplified copies of a desired target sequence even when there is mismatch between the primer and the template.

It is likely that the heterogeneity in sequence at the termini of the amplified DNA will be greatly reduced when the amplification reaction is catalyzed by *Taq* DNA polymerase rather than by the Klenow fragment. The higher temperatures used during annealing and/or extension of oligonucleotides by *Taq* DNA polymerase should suppress hybridization between mismatched primers and the target sequence, generating an amplified product that carries the "correct" oligonucleotides at its termini. However, when using mixed pools of oligonucleotides as primers for *Taq* DNA polymerase, it is essential to give some thought to both the temperature of annealing and the "ramping time" (i.e., the time required to raise the temperature of the

annealing mixture to 72°C, the temperature routinely used for polymerization). The temperature chosen for annealing should be low enough to ensure that the oligonucleotide with the lowest content of G + C in the pool has an opportunity to hybridize efficiently to its target. The ramping time should be short enough to suppress elongation of oligonucleotides that hybridize to false target sequences.

In a variation of the MOPAC technique, called the *anchored polymerase chain reaction* (Loh et al. 1989), the first strand of cDNA is synthesized in a conventional way using as a primer either oligo(dT) or a synthetic oligonucleotide complementary to the sequence of mRNA that codes for a known tract of amino acids. Although the original experiments used only a single synthetic oligonucleotide for this purpose, degenerate pools of oligonucleotides would also be expected to work well. A poly(dG) tail is then added to the 3′ termini of the resulting cDNAs with terminal transferase, and the desired product is amplified in a polymerase chain reaction primed by (1) an oligonucleotide consisting of a poly(dC) tail attached to a sequence with convenient restriction sites (termed the anchor) and (2) a single synthetic oligonucleotide (or a degenerate pool of oligonucleotides) corresponding to a known tract of amino acids in the protein of interest. If desired, these oligonucleotides can also be anchored. This simplifies cloning of the amplified product.

The central benefit of both of these methods is that a unique primer is not needed at both ends of the sequence to be amplified. Furthermore, because the resulting amplified DNAs are highly enriched for the sequences of interest, there is no need to establish or screen large cDNA libraries.

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Generation of Libraries of cDNA from Small Amounts of mRNA

The polymerase chain reaction can be used to amplify first-strand cDNA that has been synthesized in vitro by DNA-dependent RNA polymerase from mRNA templates using oligo(dT) as a primer. The addition of a homopolymeric tract of G residues to the 3′ terminus of the cDNA allows the subsequent polymerase chain reaction to be primed by oligo(dT) and oligo(dC) primers (see, e.g., Belyavsky et al. 1989). If desired, these primers can contain a potential restriction site at their 5′ termini to facilitate cloning of the amplified double-stranded cDNA into an appropriate vector. The main advantage of this method is that it allows large cDNA libraries to be established from mRNA extracted from as few as 1 or 2 mammalian cells. However, it is important to bear in mind that the amplified sequences will accumulate a high proportion of errors (0.25% if the cDNA is amplified through ~35 cycles). It is therefore essential to compare the sequences of several independent isolates of any clones of interest obtained from such amplified libraries. In addition, smaller fragments of cDNA will accumulate preferentially during the amplification process, and this method is therefore not recommended for the isolation of full-length cDNAs corresponding to mRNAs more than 1–2 kb in length.

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Generation of Large Amounts of DNA for Sequencing

Sequencing can be carried out either by the Maxam-Gilbert chemical degradation method or, more commonly, by the Sanger dideoxy-mediated chain-termination method (see Chapter 13). Three types of polymerase chain reaction are used to generate templates for sequencing by the Sanger method:

- *A conventional reaction in which both priming oligonucleotides are present in vast excess.* At the end of the reaction, the amplified DNA is desalted and excess dNTPs are removed by centrifuge-driven spin dialysis on a microconcentrator (Amicon). The amplified DNA is then sequenced using a ^{32}P-labeled oligonucleotide primer complementary to an appropriate region of the DNA. A description of this method is given on pages 14.22–14.27.

  Although this method can work well, the presence of oligonucleotide primers (remaining from the amplification reaction) can complicate the dideoxy-mediated sequencing reaction. These unlabeled primers may either compete directly with the radiolabeled primer for binding sites on the template DNA or they may generate extension products that efficiently exclude the radiolabeled oligonucleotide. These problems can be avoided by purifying the amplified DNA by gel electrophoresis (see Chapter 6).

- *A conventional reaction in which at least one of the primers is designed to include a promoter for a bacteriophage-encoded DNA-dependent RNA polymerase.* At the end of the reaction, one strand of the amplified DNA is transcribed in vitro, and the resulting RNA is sequenced using reverse transcriptase and an oligonucleotide complementary to the 3′ terminus of the RNA (Stoflet et al. 1988).

  The chief disadvantage of this method is the necessity of generating long oligonucleotides that contain bacteriophage promoters to prime the amplification reaction. When both strands of the amplified DNA are to be sequenced, each of the two oligonucleotides must contain a separate promoter sequence. The expense of generating such long oligonucleotides can become burdensome when this method is used routinely.

- *A reaction in which one of the two primers is present in limiting concentration.* After approximately 12–15 cycles of amplification, one primer is essentially depleted and the final stages of the amplification reaction therefore generate copies of only one strand of the original DNA (Gyllensten and Erlich 1988; Mihovilovic and Lee 1989). Sequencing is carried out using unlabeled primers complementary to an appropriate region of the amplified single-stranded DNA. A description of this method is given on pages 14.28–14.29.

  This method generally works well. However, to optimize the yield of single-stranded product, it is sometimes necessary to carry out several amplification reactions that contain varying amounts and ratios of the priming oligonucleotides. In addition, separate sets of amplification reactions must be set up if both strands of the amplified DNA are to be sequenced.

Analysis of Mutations

The polymerase chain reaction has been adapted in a number of ways to facilitate the identification and analysis of mutations in eukaryotic DNA. For example, deletions and insertions at defined locations can be detected by a change in the size of the amplified product. Alternatively, deletions can be recognized by their failure to amplify when one of the priming oligonucleotides maps within the deleted sequence. The polymerase chain reaction can also be useful in the identification of point mutations in the following ways:

- Hybridizing amplified DNA to radiolabeled RNA probes (Almoguera et al. 1988). Perfectly matched heteroduplexes can be distinguished from mismatched duplexes by digestion with RNAase A (see Chapter 7, pages 7.71–7.78).
- Using mixtures of synthetic oligonucleotides as primers for DNA synthesis (Gibbs et al. 1989). A DNA template is mixed with two oligonucleotides that differ in sequence by a single base and therefore compete for the same site in the target DNA. During the course of a polymerase chain reaction, the perfectly matched oligonucleotide is favored over its mismatched competitor and is therefore incorporated into the amplified DNA product. In this technique, which is called *competitive nucleotide priming*, the two oligonucleotides are included in two separate reactions. In one reaction, the first primer is radiolabeled; in the second reaction, the other primer is radiolabeled. Both reactions generate an amplified product, but only the reaction primed by the perfectly matched oligonucleotide yields a product radiolabeled to high specific activity. It is therefore a simple matter to determine which primer correctly matches the sequence in the original unamplified sample.
- Using allele-specific oligonucleotide probes to discriminate between amplification products that differ by a single base pair (Saiki et al. 1988a). This method often requires the application of a comprehensive panel of oligonucleotide probes in order to check for each possible change (see, e.g., Hirai et al. 1987; Liu et al. 1987; Rodenhuis et al. 1987; Farr et al. 1988).

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Chromosome Crawling

Although the polymerase chain reaction is used frequently to amplify DNA segments lying between the two priming oligonucleotides, it can also be used to amplify sequences that lie outside the boundary of known sequences (Ochman et al. 1988; Triglia et al. 1988). As shown in Figure 14.2, genomic DNA is digested to completion with a restriction enzyme that has no sites of cleavage within the target sequence. The fragment carrying the target sequence should be no larger than 2–3 kb in length. The DNA is then diluted and ligated under conditions that favor the formation of monomeric circles. Because *Taq* DNA polymerase works slightly more efficiently with linear DNA than with circular DNA, some workers (Triglia et al. 1988) relinearize the template DNA by digesting with a restriction enzyme that cleaves once within the known sequence of the target DNA (see Figure 14.2). No matter whether linear or circular templates are used, the regions flanking the target sequence can then be amplified in a polymerase chain reaction using oligonucleotide primers that are complementary to the 5′ termini of the target fragment. The major product of the amplification reaction is a linear double-stranded DNA molecule that consists of a head-to-tail arrangement of sequences flanking the original target DNA. The junction between the upstream and downstream sequences is marked by the presence of a restriction site for the enzyme originally used to digest the genomic DNA. This procedure, which is known as *inverse PCR* (Ochman et al. 1988), permits the rapid amplification of uncharacterized segments of DNA that immediately flank a target sequence. Inverse PCR is therefore useful as a method ("chromosome crawling" [Triglia et al. 1988]) to explore the chromosomal sequences that are contiguous to a known segment of DNA.

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FIGURE 14.2
A diagram of the major steps involved in an inverse polymerase chain reaction. The target DNA is shown as ∿∿ and the flanking sequences are shown as ▭ and ▭. Restriction sites are marked ▲ and △. Arrows show the positions of complementarity between the priming oligonucleotides and the template DNA.

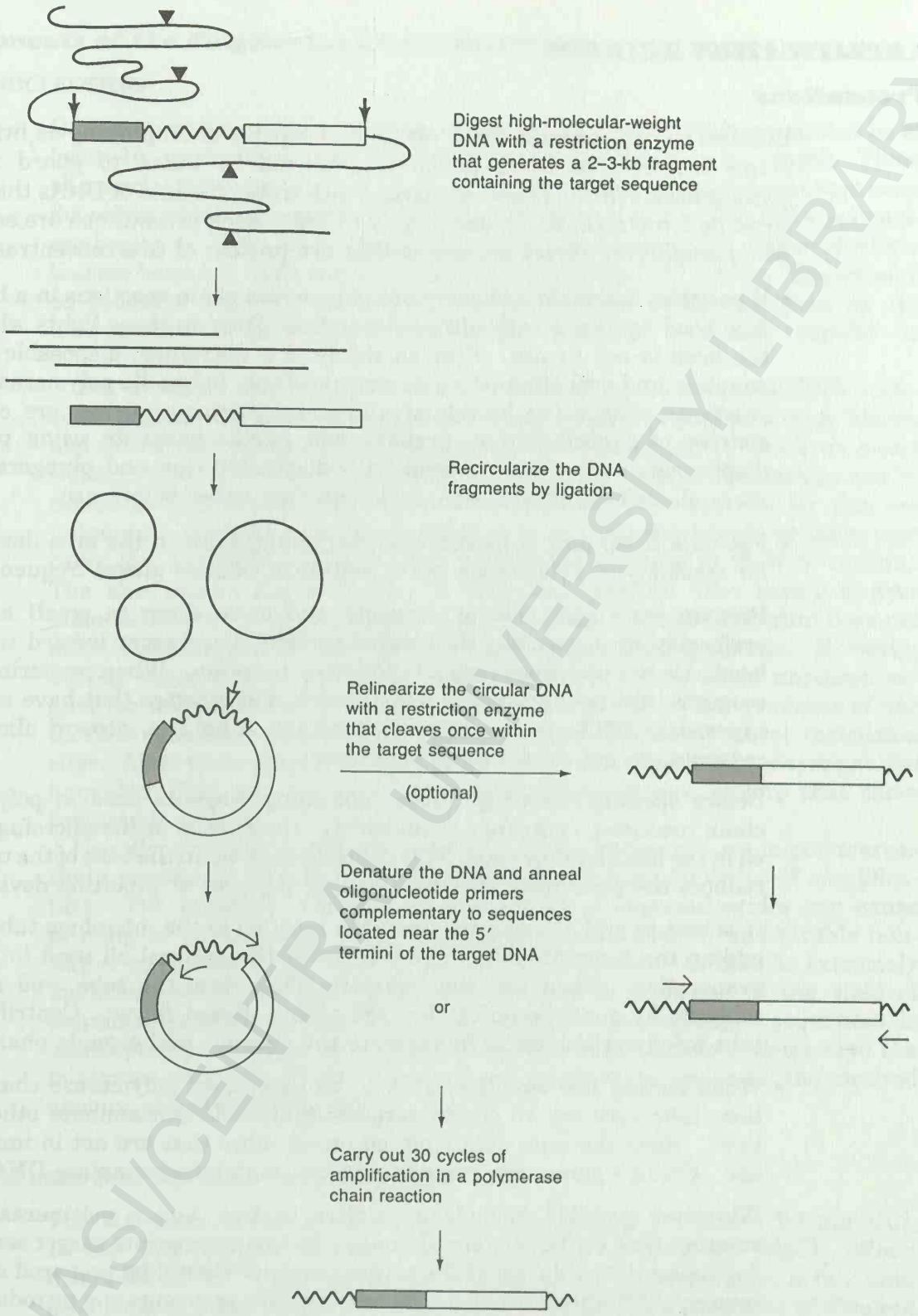
Digest high-molecular-weight DNA with a restriction enzyme that generates a 2–3-kb fragment containing the target sequence
Recircularize the DNA fragments by ligation
Relinearize the circular DNA with a restriction enzyme that cleaves once within the target sequence
(optional)
Denature the DNA and anneal oligonucleotide primers complementary to sequences located near the 5′ termini of the target DNA
or
Carry out 30 cycles of amplification in a polymerase chain reaction
The major product of the amplification reaction consists of a head-to-tail arrangement of the sequences that originally flanked the target region. The junction between the two sets of flanking sequences is marked by a restriction site that can be cleaved by the enzyme used in step 1 to digest the original high-molecular-weight DNA.

FIGURE 14.2 *(See facing page for legend.)*

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Precautions

Because the polymerase chain reaction is capable of amplifying as little as a single molecule of DNA, precautions should be taken to guard against contamination of the reaction mixture with trace amounts of DNAs that could serve as templates (Kwok and Higuchi 1989). Such precautions are essential when amplifying target sequences that are present at low concentrations.

- If possible, assemble and carry out polymerase chain reactions in a laminar flow hood equipped with ultraviolet lights. Turn on these lights whenever the hood is not in use. Keep in the hood a microfuge, disposable gloves, supplies, and sets of pipetting devices used only to handle polymerase chain reactions. Since the barrels of automatic pipetting devices are common sources of contamination, prepare and handle reagents using positive-displacement pipettes equipped with disposable tips and plungers. Autoclave all buffers, pipette tips, and centrifuge tubes before use.
- Put on a fresh pair of gloves when beginning work in the area designated for assembly of polymerase chain reactions. Change gloves frequently.
- Prepare your own sets of reagents and store them in small aliquots, preferably in a specially designated section of a freezer located near the hood. Do *not* use these reagents for other purposes. When preparing these reagents, use new glassware, plasticware, and pipettes that have not been exposed to DNAs in use in the laboratory. After use, discard aliquots of reagents; do not return them to storage.
- Before opening microfuge tubes containing reagents used in polymerase chain reactions, centrifuge them briefly (10 seconds) in the microfuge located in the laminar flow hood. This deposits the fluid in the base of the tube and reduces the possibility of contamination of gloves or pipetting devices.
- It is best to add *all* components of the reaction to the microfuge tube *before* adding the template DNA. This includes the mineral oil used to prevent evaporation. Then add the template DNA, close the tube, and mix the contents by gently tapping the side with a gloved finger. Centrifuge the tube briefly (10 seconds) to separate the aqueous and organic phases.
- When adding the template DNA to an assembled polymerase chain reaction, take care not to create aerosols that could contaminate other reactions. Keep the tops tightly closed on all tubes that are not in immediate use. Change gloves after handling tubes containing template DNA.
- Whenever possible, include a positive control (i.e., a polymerase chain reaction that contains a small amount of the appropriate target sequence). An appropriate dilution of the target sequence should be prepared ahead of time in a different area of the laboratory. This prevents the introduction of concentrated solutions of target DNA into the area of the laboratory set aside for polymerase chain reactions.
- Always include a control that contains all the components of the polymerase chain reaction except the template DNA. Assemble this control after all other polymerase chain reactions have been set up.

Components of the Polymerase Chain Reaction

OLIGONUCLEOTIDES

Oligonucleotides used for priming the polymerase chain reaction should be at least 16 nucleotides and preferably 20–24 nucleotides in length. These oligonucleotides are too short to form stable hybrids at the temperature used for polymerization (usually 72°C). It is assumed that the *Taq* DNA polymerase begins to work, albeit sluggishly, as soon as the priming oligonucleotides become bound to their templates at low temperature (37–55°C). The extended products are long enough to remain attached to the template as the temperature of the polymerase chain reaction is raised rapidly to 72°C.

The products of polymerase chain reactions are blunt-ended DNA molecules that can be phosphorylated by bacteriophage T4 polynucleotide kinase and cloned into standard vectors by conventional techniques. However, many workers have found that the efficiency with which amplified molecules can be cloned in this way is much lower than normal. The reasons for this are unknown. A solution to the problem is to use oligonucleotide primers that carry the recognition sequence for a restriction enzyme at their 5′ termini. The site chosen should be for a restriction enzyme that rarely cleaves mammalian DNA. The presence of unpaired bases at the 5′ termini does not affect the ability of the oligonucleotides to prime DNA synthesis. However, during successive cycles of the amplification reaction, these unpaired sequences are converted to double-stranded DNA. The final products of the reaction therefore consist of target sequences flanked by novel restriction sites. After these sites have been cleaved with the appropriate enzyme(s), the amplified DNA can be cloned with high efficiency into vectors that carry compatible termini.

Usually, oligonucleotides are used at a concentration of 1 μM in polymerase chain reactions. This is usually sufficient for at least 30 cycles of amplification. The presence of higher concentrations of oligonucleotides can cause priming at ectopic sites, with consequent amplification of undesirable nontarget sequences. Conversely, the polymerase chain reaction is extremely inefficient when the concentration of primers is limiting. If the yield of amplified product is poor or if contamination by nontarget sequences is unacceptably high, set up a series of control experiments to determine the minimum amounts of the two primers required to generate the desired amount of amplified product.

BUFFERS USED FOR POLYMERASE CHAIN REACTIONS

The standard buffer for polymerase chain reactions contains 50 mM KCl, 10 mM Tris·Cl (pH 8.3 *at room temperature*) and 1.5 mM $MgCl_2$. When incubated at 72°C, the pH of the reaction drops by more than a full unit, producing a buffer whose pH is approximately 7.2. The presence of divalent cations is critical. Magnesium ions are superior to manganese, and calcium ions are ineffective (Chien et al. 1976). Because the optimal concentration of Mg^{++} is quite low (1.5 mM), it is important that the preparation of template DNA does not contain high concentrations of chelating agents such as EDTA or of negatively charged ionic groups such as phosphates. DNAs to be used as

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templates should therefore be dissolved in 10 mM Tris · Cl (pH 7.6), 0.1 mM EDTA (pH 8.0).

The standard buffer works well for a wide range of templates and oligonucleotide primers, but it may not be optimal for any particular combination (see, e.g., Krawetz et al. 1989). The conditions given should therefore be regarded as a point of departure to explore modifications and potential improvements. In particular, the concentration of Mg^{++} should be optimized whenever a new combination of target and primers is first used or when the concentration of dNTPs or primers is altered. dNTPs are the major source of phosphate groups in the reaction, and any change in their concentration affects the concentration of available Mg^{++}. We recommend setting up a set of reactions containing fixed concentrations of Tris · Cl (10 mM) and KCl (50 mM) and varying concentrations of $MgCl_2$ (0.05–5 mM in 0.5 mM increments). After completion of the reactions, compare the yields of the amplified products by electrophoresis through an agarose gel in the presence of ethidium bromide.

Taq DNA POLYMERASE

Two forms of *Taq* DNA polymerase are now available: the native enzyme purified from *Thermus aquaticus* and a genetically engineered form of the enzyme synthesized in *E. coli* (AmpliTaq™). Both forms of the polymerase carry a 5′→3′ polymerization-dependent exonuclease activity, but they lack a 3′→5′ exonuclease activity (S. Stoffel, pers. comm.). The properties of the two polymerases are essentially equivalent (see Chapter 5, page 5.50), and they can be used interchangeably in polymerase chain reactions. Approximately 2 units of either of the enzymes are required to catalyze a typical polymerase chain reaction. Addition of excess enzyme may lead to amplification of nontarget sequences.

DEOXYRIBONUCLEOSIDE TRIPHOSPHATES

dNTPs are used at saturating concentrations (200 μM for each dNTP). A stock solution of dNTPs (50 mM) should be adjusted to pH 7.0 with 1 N NaOH to ensure that the pH of the final reaction does not fall below 7.1.

Prepared solutions of dNTPs sold by commercial manufacturers are generally not adjusted to pH 7.0.

TARGET SEQUENCES

DNA containing the target sequences can be added to the polymerase chain reaction mixture in a single- or double-stranded form. Although the size of the DNA is generally not a critical factor, amplification is improved if very-high-molecular-weight DNAs (e.g., genomic DNAs) are digested with a rarely cutting restriction enzyme (e.g., *Sal*I or *Not*I). Target sequences are amplified slightly less efficiently when they are carried in closed circular DNAs rather than in linear DNAs. It is therefore preferable to linearize plasmid DNAs before they are used as templates in polymerase chain reactions. The concentration of target sequences in the template DNA

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obviously varies according to circumstances and is often not under the control of the experimenter. However, it is worthwhile setting up a series of control reactions that contain decreasing amounts of known target sequences (1 ng, 0.1 ng, 0.001 ng, etc.) to check that the amplification reaction is working at the required sensitivity.

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1. In a sterile 0.5-ml microfuge tube, mix in the following order:

sterile H_2O	30 μl
10 × amplification buffer	10 μl
mixture of four dNTPs, each at a concentration of 1.25 mM	16 μl
primer 1 (in 5 μl of H_2O)	100 pmoles
primer 2 (in 5 μl of H_2O)	100 pmoles
template DNA (up to 2 μg, depending on the concentration of target sequences)	
H_2O to a final volume of 100 μl	

10 × Amplification buffer

500 mM KCl
100 mM Tris · Cl (pH 8.3 at room temperature)
15 mM $MgCl_2$
0.1% gelatin

The standard conditions given above work well for a wide range of templates and oligonucleotide primers, but they may not be optimal for any particular combination. Methods to optimize the reaction conditions are discussed on pages 14.14–14.17.

The pH of the mixture of dNTPs should be 7.0 (see page 14.16).

For amplification of single sequences from mammalian genomic DNA, use 0.2–2 μg of DNA. The reaction mixture will then contain approximately 0.03–0.3 pg of a target sequence 500 bp in length. For amplification of target DNA cloned in a plasmid vector (e.g., for DNA sequencing), add 20 ng of linearized plasmid DNA.

Because the optimal concentration of Mg^{++} in the reaction is quite low, DNAs to be used as templates should be dissolved in 10 mM Tris · Cl (pH 7.6), 0.1 mM EDTA (pH 8.0) (see pages 14.15–14.16).

2. Heat the reaction mixture for 5 minutes at 94°C to denature the DNA completely.

3. While the mixture is still at 94°C, add 0.5 μl of *Taq* DNA polymerase (5 units/μl; Perkin Elmer Cetus N801-0046).

Taq DNA polymerase is supplied in a storage buffer containing 50% glycerol. This solution is very viscous and is difficult to pipette with accuracy. The best method is to centrifuge the tube containing the enzyme at 12,000*g* for 10 seconds at 4°C in a microfuge and then to withdraw the required amount of enzyme using a positive-displacement pipetting device. Automatic pipetting devices are not recommended for handling reagents used in polymerase chain reactions (see page 14.14).

4. Overlay the reaction mixture with 100 μl of light mineral oil (Sigma M-3516 or equivalent). This prevents evaporation of the sample during repeated cycles of heating and cooling.

5. Carry out amplification as described below. Typical conditions for denaturation, annealing, and polymerization are as follows:

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Cycle	Denaturation	Annealing	Polymerization
First cycle	5 minutes at 94°C	2 minutes at 50°C	3 minutes at 72°C
Subsequent cycles	1 minute at 94°C	2 minutes at 50°C	3 minutes at 72°C
Last cycle	1 minute at 94°C	2 minutes at 50°C	10 minutes at 72°C

The denaturation step is omitted after the last cycle of amplification and the samples are transferred to −20°C for storage.

Timing of individual steps should begin only after the reaction mixtures have reached the required temperatures. Between 30–60 seconds are required for reaction mixtures to reach the desired temperature after a temperature shift. The length of the lag depends on several factors, including the type of tube used, the volume of the reaction mixture, the source of the heat (water bath or heating block), and the temperature differential between successive steps. It is important to adjust the length of the incubation steps to compensate for these lags.

The temperature (50°C) chosen for annealing of the oligonucleotide primers to the target DNA is a compromise. Amplification is more efficient if annealing is carried out at lower temperatures (37°C), but the amount of mispriming is significantly increased (Saiki et al. 1988b). At higher temperatures (55°C), the specificity of the amplification reaction is increased, but its overall efficiency is reduced. Ideally, a series of control reactions should be set up to determine the annealing temperature that gives optimal results for a given amplification reaction.

Polymerase chain reactions can be automated with a thermal cycler (available from Perkin Elmer Cetus or Ericomp). These machines, although expensive, allow the individual cycles of the reactions to be standardized and relieve the experimenter of the burden of transferring tubes from one temperature to another for many hours.

The longer the distance between the oligonucleotide primers, the longer the time required for the complete synthesis of the entire sequence of the target sequence. The times given above are optimized for a target sequence approximately 500 nucleotides in length.

The number of amplification cycles depends on the concentration of target DNA in the reaction mixture. At least 25 cycles are required to amplify single-copy target sequences in mammalian genomic DNA to the point where they can be detected by direct examination of agarose or polyacrylamide gels. However, the amount of *Taq* DNA polymerase usually becomes limiting after 25–30 cycles of amplification (i.e., after an $\sim 10^6$-fold amplification has been obtained). If further amplification is required, a sample of the amplified DNA should be diluted 1000- to 10,000-fold and used as the template for further rounds of synthesis in a fresh polymerase chain reaction.

6. Withdraw a sample of the amplified DNA from the reaction mixture and analyze it by gel electrophoresis, Southern hybridization, or DNA sequencing. If necessary, the oil can be removed from the sample by extraction with 150 μl of chloroform. The aqueous phase, which contains the amplified DNA, forms a micelle near the meniscus. This micelle can be transferred to a fresh tube with an automatic micropipettor.

Amplification of DNA Generated by Reverse Transcription of mRNA

The first strand of cDNA generated by reverse transcription of mRNA can be used as a template for polymerase chain reactions as described below.

1. Set up a reaction to synthesize the first strand of cDNA:

10 × amplification buffer	2 μl
mixture of four dNTPs, each at a concentration of 10 mM	2 μl
oligo$(dT)_{12-18}$ (100 μg/ml)	1 μl
placental RNAase inhibitor	20 units
mRNA	1–2 μg
50 mM $MgCl_2$	1 μl
H_2O to 20 μl	
murine reverse transcriptase	100–200 units

Incubate the reaction for 30 minutes at 37°C.

10 × Amplification buffer

500 mM KCl
100 mM Tris · Cl (pH 8.3 at room temperature)
15 mM $MgCl_2$
0.1% gelatin

10–50 pmoles of a synthetic oligonucleotide complementary to a desired sequence of the mRNA may be used in place of oligo(dT) as a primer.

Bovine serum albumin (Fraction V, Sigma; final concentration 100 μg/ml) may be substituted for gelatin in the reverse transcription reaction.

The amount of $MgCl_2$ in the amplification buffer needs to be supplemented with additional $MgCl_2$ in the reverse transcriptase reaction mixture because reverse transcriptase requires higher concentrations of divalent cations than does *Taq* DNA polymerase.

A method for rapidly preparing RNA suitable for chain reactions is given on p. 14.33.

2. Inactivate the reverse transcriptase by heating the reaction to 95°C for 5 minutes.

This step improves the efficiency of amplification and the "cleanness" of the product.

3. Add:

"upstream" oligonucleotide primer (i.e., primer complementary to the original mRNA)	10–50 pmoles
"downstream" oligonucleotide primer (i.e., primer complementary to the first strand of cDNA)	10–50 pmoles
1 × amplification buffer to a final volume of 79 μl	
Taq DNA polymerase (5 units/μl) (Perkin Elmer Cetus N801-0046)	1–2 units

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The total amount of "upstream" primer in the amplification reaction should be 10–50 pmoles. This includes any "upstream" oligonucleotide primer that is used instead of oligo(dT) to prime the synthesis of the first strand of cDNA (see note to step 1).

The presence of excess oligonucleotide primers in the reaction often leads to amplification of undesirable nontarget sequences. It is therefore desirable to carry out preliminary experiments to determine the minimum amounts of the two primers that are required for satisfactory amplification.

4. Overlay the reaction mixture with 100 μl of light mineral oil (Sigma M-3516 or equivalent). This prevents evaporation of the sample during repeated cycles of heating and cooling.

5. Carry out the desired number of cycles of amplification as described on pages 14.18–14.19, step 5.

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SEQUENCING AMPLIFIED DNA BY THE SANGER DIDEOXY-MEDIATED CHAIN-TERMINATION METHOD

Protocol I: Sequencing Amplified DNA with Radiolabeled Oligonucleotide Primers

The quality of the DNA sequence generated by this method depends on the efficiency with which the oligonucleotides used to prime the PCR amplification are removed from the amplified DNA. These unlabeled primers may either compete directly with the radiolabeled primer for binding sites on the template DNA or generate extension products that efficiently exclude the radiolabeled oligonucleotide. It is also necessary to remove all of the dNTPs from the amplified DNA, because these will serve as substrates in the subsequent chain-termination reactions. Both dNTPs and oligonucleotides can be removed by spin dialysis in microcentrators (see below) or by purifying the amplified DNA by polyacrylamide or agarose gel electrophoresis (see Chapter 6). Many workers have found that better results are obtained when an end-labeled nested sequencing primer that anneals to the amplified DNA within the borders defined by the 3′ termini of the amplification primers is used rather than a radiolabeled amplification primer.

REMOVAL OF OLIGONUCLEOTIDES AND EXCESS dNTPs FROM AMPLIFIED DNA BY SPIN DIALYSIS

During spin dialysis, the solvent, together with low-molecular-weight solutes, is centrifuged through an anisotropic, hydrophilic, nonabsorbent, porous membrane. Macromolecules larger than the membrane pores are retained in the sample reservoir in a small volume of solvent. Following removal of the solutes, the concentrated sample is reconstituted to the original volume using the desired solvent. This process is repeated until the concentration of the original solutes is reduced to acceptable levels.

Spin dialysis can be conveniently carried out in disposable microconcentrators (Amicon). The microconcentrators are supplied with membranes of several different porosities that are characterized by a nominal molecular-weight cut-off. For removal of oligonucleotides and dNTPs, Centricon 30 microconcentrators, which retain proteins with a molecular weight of more than 30,000, are routinely used. As far as we are aware, there has been no systematic study of the efficiency with which oligonucleotides of different lengths are removed by these microconcentrators. It is clear, however, that oligonucleotides up to 24 nucleotides in length can be eliminated from amplified DNA by four sequential cycles of centrifugation and dilution (see, e.g., Mihovilovic and Lee 1989). Longer oligonucleotides are best removed by purifying the amplified DNA by gel electrophoresis.

1. Carry out a conventional amplification reaction as described on pages 14.18–14.19. After purifying the amplified DNA by extraction with chloroform (step 6), transfer 50 μl of the solution containing the amplified DNA to a fresh microfuge tube.

2. Add 0.75 ml of H_2O to the amplified DNA, and transfer the sample to the reservoir chamber of a Centricon 30 microconcentrator (see Figure 14.3). These concentrators are easily distinguished from other Centricon concentrators by virtue of their *clear* plastic base. Do not touch the membrane with the tip of the pipette when loading the microconcentrator.

3. Replace the cap (also called the retentate cup) on the microconcentrator, and place the entire device (retentate cup/sample reservoir/filtrate cup) in a *fixed-angle* rotor (Sorvall SS34 or equivalent) equipped with adapters or carriers for 17-mm × 100-mm centrifuge tubes.

   The microconcentrator should be inserted into the centrifuge with the filtrate cup towards the bottom of the rotor. A microconcentrator filled with an equivalent amount of fluid should be used as a counterbalance.

4. Centrifuge the loaded microconcentrator at 3000*g* (~6500 rpm in a Sorvall SS34 rotor) for 6 minutes at room temperature.

5. Remove the microconcentrator from the centrifuge, and take off the retentate cup. Add 0.75 ml of H_2O to the sample reservoir, replace the retentate cup, and recentrifuge as described in step 4.

6. Repeat step 5 twice more.

   At the end of the fourth centrifugation, the filtrate cup should be almost filled (total capacity of the filtrate cup is 3.4 ml when held at an angle of 34° in a Sorvall SS34 rotor). The volume of the sample should be approximately 50 μl.

7. Remove the microconcentrator from the centrifuge, and discard the filtrate cup.

8. Invert the unit, and replace it in the centrifuge (i.e., the retentate cup should now be placed towards the bottom of the rotor). Centrifuge at 1000*g* (~2500 rpm in a Sorvall SS34 rotor) for 2 minutes at room temperature.

9. Remove the microconcentrator from the centrifuge, and take off the retentate cup. Discard the rest of the device. Transfer the fluid in the retentate cup to a fresh microfuge tube.

10. Recover the amplified DNA by ethanol precipitation. To the sample of DNA, add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol. Store the tube in an ice bath for 15 minutes, and then recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge.

11. Carefully discard the supernatant, and wash the pellet with 1 ml of 70% ethanol at room temperature, recentrifuging if necessary. Discard the supernatant, and store the open tube at room temperature until all of the ethanol has evaporated. Redissolve the DNA in 100 μl of TE (pH 7.6).

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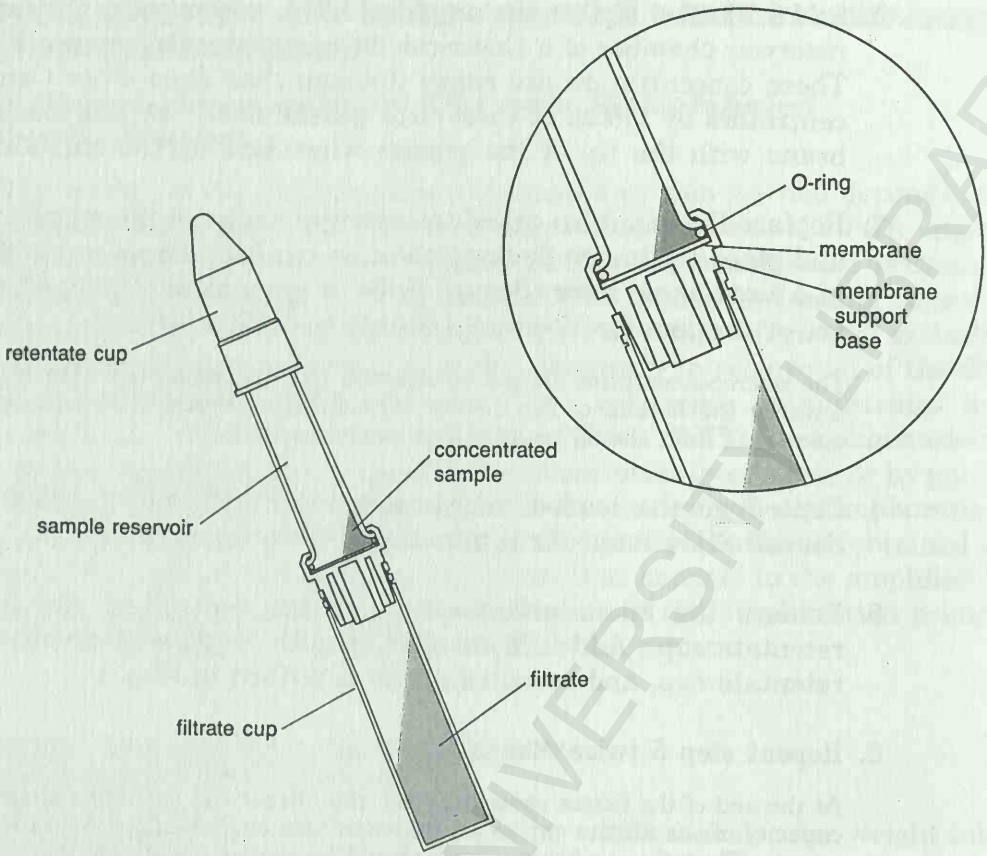

FIGURE 14.3
Centricon components. Each disposable Centricon unit includes a concentrator, filtrate cup, and retentate cup. The concentrator consists of a type YM membrane and an O-ring, permanently sealed between the sample reservoir and its support base. (Redrawn, with permission, from Amicon.)

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RADIOLABELING OF THE OLIGONUCLEOTIDE SEQUENCING PRIMER

1. In a sterile microfuge tube, mix:

oligonucleotide (20 μM)	2.5 μl
10 × bacteriophage T4 polynucleotide kinase buffer	1.0 μl
[γ-^{32}P]ATP (5000 Ci/mmole; 10 mCi/ml in aqueous solution)	1.0 μl
H_2O to 9 μl	
bacteriophage T4 polynucleotide kinase (8 units/μl)	1.0 μl

Incubate the reaction for 30 minutes at 37°C.

10 × Bacteriophage T4 polynucleotide kinase buffer

500 mM Tris · Cl (pH 7.6)
100 mM $MgCl_2$
50 mM dithiothreitol
1 mM spermidine HCl
1 mM EDTA (pH 8.0)

2. Stop the reaction by heating to 68°C for 10 minutes.

3. Use Whatman DE-81 filters to check the incorporation of ^{32}P into the oligonucleotide (see Appendix E).

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1. In a sterile microfuge tube, mix:

^{32}P-labeled oligonucleotide (5 pmoles/μl)	1.0 μl
amplified DNA	10.0 μl
10 × annealing buffer	1.5 μl
H_2O	2.5 μl

10 × Annealing buffer

500 mM Tris · Cl (pH 8.0)
500 mM KCl
50 mM $MgCl_2$
100 mM dithiothreitol

2. Close the top of the tube, and incubate the reaction mixture in a boiling-water bath for 2 minutes.

3. Transfer the tube to a microfuge, and centrifuge at 12,000*g* for 5 seconds at room temperature.

4. Immediately transfer the tube to a water bath set at 37°C. Incubate for 5 minutes.

5. During the incubation, set up sequencing reactions in four tubes labeled ddA, ddC, ddG, and ddT. Place 2 μl of the corresponding ddNTP reaction mixture (see Table 14.1) in each tube. Transfer the tubes to a water bath set at 37°C.

6. At the end of the 5-minute incubation, add 1 μl (~200 units) of reverse transcriptase (cloned murine enzyme; BRL 8025SA) to the tube containing the amplified DNA.

7. Add 3 μl of the amplified DNA to each of the four sequencing reactions.

8. Incubate the reactions for 30 minutes at 37°C.

9. Add 6 μl of formamide-dye mixture to each tube.

TABLE 14.1 Preparation of ddNTP Reaction Mixtures in Annealing Buffer

ddNTP mixture	dATP (μM)	dCTP (μM)	dGTP (μM)	dTTP (μM)	ddNTP (μM)
ddATP	10	100	100	100	2.5 ddATP
ddCTP	10	20	100	100	5 ddCTP
ddGTP	10	100	20	100	6 ddGTP
ddTTP	10	100	100	40	14 ddTTP

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Formamide-dye mixture

98% formamide
0.025% bromophenol blue
0.025% xylene cyanol FF

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C. Several companies sell formamide distilled and packaged under nitrogen that need not be purified before use (e.g., BRL 5515UA).

10. Heat the tubes to 100°C for 5 minutes, and then analyze 4 μl of each sequencing reaction by electrophoresis through a polyacrylamide gel as described in Chapter 13, pages 13.45–13.58.

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Protocol II: Sequencing Single-stranded DNA Templates Generated by Asymmetric Amplification

In this method (Gyllensten and Erlich 1988; Mihovilovic and Lee 1989), amplification reactions are set up that contain vastly different molar ratios of the two oligonucleotide primers. During the first 10–15 cycles of amplification, the product is exclusively double-stranded DNA, which accumulates at an approximately exponential rate. After 12–15 cycles of amplification, the concentration of one of the primers becomes limiting, and the rate of production of double-stranded DNA declines markedly. However, the second oligonucleotide continues to prime the synthesis of single-stranded DNA that is complementary to a particular sequence of one of the two strands of the template DNA. Although the single-stranded product accumulates only at a linear rate during successive rounds of synthesis and denaturation, it reaches concentrations that are more than adequate for conventional dideoxy-mediated sequencing.

1. Set up amplification reactions as described on page 14.18, except use unequal amounts of the two oligonucleotide primers that are added to the reaction. A typical asymmetric amplification reaction might contain 100 pmoles of one oligonucleotide and 1 pmole of the other. Amplification is normally carried out for 25 cycles.

   To ensure that the two primers are present in the correct ratio, some workers prefer to set up several amplification reactions containing constant amounts of one of the two primers (100 pmoles) and different amounts of the other (0.25 pmole, 1 pmole, and 4 pmoles). The amount of single-stranded product in each of the reactions can be estimated at the end of the amplification by agarose gel electrophoresis followed by Southern hybridization (Gyllensten and Erlich 1988).

   Asymmetric amplification works best when the concentration of target sequences in the template DNA is quite high. For example, excellent yields of single-stranded DNA (~300 ng) are obtained after 25 cycles of amplification of a 1-kb target DNA in a reaction containing 10 ng of a bacteriophage λ recombinant DNA. However, a greatly reduced yield of single-stranded product is obtained when total mammalian DNA (2 μg) is used as the template for asymmetric amplification. This is almost certainly because insufficient double-stranded target DNA is synthesized during the first stage of the reaction. This problem can usually be rectified by increasing the amounts of the two primers to 200 pmoles and 2 pmoles and increasing the number of amplification cycles to 35, with *fresh Taq* DNA polymerase being added after the 30th cycle. Alternatively, the asymmetric amplification reaction can be divided into two separate stages. The first stage is a completely conventional polymerase chain reaction, carried out with high concentrations of both primers, that generates large amounts of double-stranded target DNA. Between 5 ng and 20 ng of this DNA is then used as the template in an asymmetric amplification reaction set up as described in this protocol.

   To determine the sequence of both strands of the DNA of interest, it is necessary to set up separate sets of asymmetric amplification reactions that contain limiting concentrations of the forward and reverse primers. Often, the yield of single-stranded DNA differs substantially in the separate reactions, presumably because of variation in the efficiency with which the *Taq* DNA polymerase copies complementary DNA templates.

2. Remove excess oligonucleotide primers and dNTPs by four cycles of spin dialysis as described on pages 14.22–14.24. However, after ethanol precipitation (steps 10 and 11), redissolve the DNA in 10 μl (rather than 100 μl) of TE (pH 7.6).

3. Set up a series of trial dideoxy-mediated sequencing reactions (see Chapter 13, page 13.61) that contain different amounts (0.5–5.0 μl) of the single-stranded template DNA and a constant amount (1 μM) of a sequencing primer. The primer used for sequencing can be either the oligonucleotide primer used in limiting concentration during the amplification reaction or an oligonucleotide primer that is complementary to an internal sequence of the amplified single-stranded template. Compare the quality of the sequence obtained from each reaction, and, if necessary, set up a series of additional reactions containing the optimum ratio of single-stranded template to sequencing primer.

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QUANTITATION OF INITIAL CONCENTRATION OF TARGET SEQUENCES

Although the polymerase chain reaction is used extensively to amplify and detect minute quantities of nucleic acids, it has not been used routinely to quantitate the concentration of the sequences of interest in the original sample of nucleic acids. This is because the efficiency of the amplification process is greatly affected by small differences in any one of a number of variables. These include (1) the quality and concentrations of the reagents used (*Taq* DNA polymerase, dNTPs, oligonucleotide primers, and template nucleic acid); (2) the conditions used for denaturation, annealing, and primer extension; (3) the rate at which the temperature is changed from one step to another within each amplification cycle; and (4) the priming efficiency of the oligonucleotides, which is affected by several variables, including the propensity of the oligonucleotides to form self-priming hairpin loops. So sensitive are polymerase chain reactions to small differences in conditions that sets of ostensibly identical amplification reactions can vary up to tenfold in final yield. It is therefore impossible to draw firm conclusions about the initial concentration of the target sequence from the yield of amplified product in a standard polymerase chain reaction. Recently, however, G. Gilliland, S. Perrin, and H.F. Bunn (pers. comm.) have devised a method to measure the overall efficiency of individual polymerase chain reactions. Knowing the amount of amplified products generated, it is then a simple matter to calculate the original concentration of the sequences of interest. This method was originally developed to quantitate low-abundance mRNAs. However, it can easily be adapted for use with single- or double-stranded DNA.

The method involves coamplification of two templates: the desired target sequence and a control template that is added in known amounts to a series of amplification reactions. Although the control template uses the same primers as the target sequence, it must be distinguishable from the target sequence following amplification. For example, the control template might be an RNA generated by in vitro transcription of a mutated version of the target sequence that either contains a novel restriction site or lacks a naturally occurring site. The amplified products of the control and target sequences can then be differentiated by restriction enzyme digestion. Alternatively, the control template might differ from the target sequence in size, for example, by deletion of a small segment of the sequences that lie between the two oligonucleotide primers. When the aim of the experiment is to measure the concentration of mRNA, the best control template is a transcript that can be copied by reverse transcriptase and then coamplified with the target sequence. When measuring the concentration of DNA, the best control template is a fragment of DNA. In either case, the control template should differ from the target sequence as little as possible in order to minimize systematic differences in the efficiency of amplification. Because the control template and target sequences are present in the same amplification reaction and use the same primers, the effect of the variables mentioned earlier is nullified. The relative amounts of the two amplified products therefore reflect the relative concentrations of the control and target sequences in the original reaction mixture.

1. Using the guidelines given above, design and prepare a control template suitable for the task at hand. Measure the concentration of the control template as carefully as possible, preferably by spectrophotometry. Alternatively, estimate the amount of control template by ethidium-bromide-mediated fluorescence (see Appendix E).

2. Make a series of tenfold dilutions (in water) containing from 10 ng/ml to 1 fg/ml of the control template. After use, these dilutions should be stored at −70°C for use in step 6.

   It is important to use water rather than TE as the diluent so as to avoid altering the concentration of Mg^{++} in the polymerase chain reaction.

3. Set up a series of amplification reactions as described on page 14.18 (DNA templates) or page 14.20 (RNA templates). In addition to the usual ingredients, this series of reactions should contain:

tenfold dilutions of control template (step 2)	10 μl
[α-^{32}P]dCTP (sp. act. 3000 Ci/mmole)	10 μCi

   Do not reduce the concentration of unlabeled dCTP in the reaction mixture to increase the specific activity of the precursor pool. There is a danger that the concentration of the nucleotide could become limiting at late stages in the amplification reaction.

4. Carry out a series of polymerase chain reactions for the appropriate number of cycles as described on pages 14.18–14.19, step 5. Analyze the products of the reactions as described in step 5, a or b, below.

5. a. When using a control template that differs from the target sequence in size, analyze the sizes of the amplified products in a 20-μl aliquot of each of the reactions by gel electrophoresis and autoradiography. Recover the amplified bands of the control template and target sequences from the gel (see Chapter 6), and measure the amount of radioactivity in them in a liquid scintillation counter. Calculate the relative amounts of the two radiolabeled DNAs in each of the polymerase chain reactions. Remember to correct the amount of radioactivity to allow for differences in the molecular weights of the two radiolabeled DNAs.

   b. When using a control template that contains a novel restriction site or lacks a naturally occurring site, heat the samples to 94°C for 5 minutes following the final round of amplification. Allow the samples to cool to room temperature, and then digest a 20-μl aliquot of each of the reactions with the appropriate restriction enzyme. Analyze the sizes of the amplified DNA fragments by gel electrophoresis and autoradiography, and quantitate the amount of radioactivity in each of them as described in step 5a.

6. Examine the results to determine the concentration of control template that yields approximately the same amount of amplified product as the target sequence. Set up a second series of amplification reactions contain-

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ing a narrower range of concentrations of control template. In addition to the usual ingredients, this series of reactions should contain:

incremental dilutions of control template (1:10, 2:10, 3:10, 4:10, etc.)	10 μl
[α-^{32}P]dCTP (sp. act. 3000 Ci/mmole)	10 μCi

It is best to generate this series of dilutions from the appropriate original tenfold dilution of the control template (step 2).

Do not reduce the concentration of unlabeled dCTP in the reaction mixture to increase the specific activity of the precursor pool. There is a danger that the concentration of the nucleotide could become limiting at late stages in the amplification reaction.

7. Carry out a series of polymerase chain reactions for the appropriate number of cycles as described on pages 14.18–14.19.

8. Analyze the radiolabeled products of the amplification reactions as described in step 5, a or b. For each amplification reaction, measure the ratio of the yield of amplified control template to the yield of amplified target sequence. Plot this ratio against the amount of control template added to each amplification reaction. From the resulting straight line, determine the equivalence point (i.e., the amount of control template that gives exactly the same quantity of amplified product as the target sequence in the reaction). Calculate the concentration of the target sequence in the original sample.

Notes

i. The aim of the denaturation and reannealing step (step 5b) is to allow efficient formation of heteroduplexes consisting of one strand of control template and one strand of target sequence. When equal concentrations of the two templates are present in the original reaction mixture, the reannealed products will consist of 50% heteroduplexes and 25% of each type of homoduplex. Only one of the two types of homoduplex will be susceptible to digestion with the appropriate restriction enzyme. Thus, when the original reaction mixture contains equal quantities of target and control templates, the ratio of target to control products in the final mixture of reannealed products will be 3:1. Which of the two types of homoduplex will be susceptible to cleavage depends on whether the control template carries an extra restriction site or lacks a site that is present in the target sequence.

Heteroduplex formation is far less efficient when the target and control templates differ significantly in size. For example, no heteroduplex formation can be observed between a target sequence of 300 nucleotides and a control template of 250 nucleotides. Under these circumstances, the ratio of target to control products in the final mixture of reannealed products will be 1:1 when the original reaction mixture contains equal quantities of target and control templates.

ii. This method can be used to quantitate <1 pg of target sequence and is accurate enough to distinguish twofold differences in concentration of

target sequence. The method is also sensitive enough to detect target sequences in RNA extracted from as few as 10–50 mammalian cells. RNA suitable for use in a cDNA-driven polymerase chain reaction may be extracted from small numbers of cells as follows:

a. Pellet the cells (10–100 cells) by centrifugation for 5 seconds at 4°C in a microfuge.

b. Remove the supernatant by aspiration. Add 10–20 μl of an ice-cold lysis solution of 0.5% Nonidet P-40 (NP-40), 10 mM Tris · Cl (pH 8.0), 10 mM NaCl, 3 mM $MgCl_2$. Vortex the tube very gently to disperse the cells throughout the lysis solution.

c. Store the tube for 5 minutes on ice, and then centrifuge at 12,000*g* for 2 minutes at 4°C in a microfuge. Use the supernatant directly as the template in a cDNA-driven polymerase chain reaction as described above.

This method works well for cells such as lymphocytes. However, it may be necessary to increase the concentration of NP-40 (up to 2%) when extracting RNA from other types of mammalian cells.

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15
Site-directed Mutagenesis of Cloned DNA

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A variety of methods have been developed for introducing mutations into cloned genes, all of which involve the use of enzymes and chemicals that cleave, degrade, or synthesize DNA (for reviews, see Shortle et al. 1981; Itakura et al. 1984; Botstein and Shortle 1985; Craik 1985; Smith 1985). These mutations can be classified into several broad categories:

- Simple deletions or insertions
- Systematic deletions, insertions, or substitutions of clusters of bases
- Substitutions of single bases

The generation of each type of mutation can be approached in a number of different ways, as summarized and discussed in detail below.

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Generation of Deletions and Insertions

SIMPLE DELETIONS OR INSERTIONS

The simplest mutations to make are deletions of segments of foreign DNA sequences cloned in plasmid vectors. If the restriction map or, better yet, the complete nucleotide sequence of the foreign DNA is known, it may be possible to construct a set of deletion mutants by digestion with the appropriate restriction enzymes. For example, the DNA sequences between restriction sites that occur only in the foreign DNA (and not in the vector) can be deleted by closing the digested plasmid DNA by ligation. If the restriction enzyme(s) leave blunt ends or compatible cohesive termini, ligation can be carried out directly. However, if incompatible cohesive termini are generated, the Klenow fragment of *E. coli* DNA polymerase I, bacteriophage T4 DNA polymerase, nuclease S1, or mung-bean nuclease must be used to produce blunt-ended molecules that can then be recircularized with DNA ligase. In many cases, however, restriction enzymes that cleave at appropriate locations within the cloned foreign DNA also cleave within essential sequences of the vector. It is then necessary to consider more inconvenient approaches, such as partial digestion, recloning into another vector, or ligations involving three or more DNA fragments. Similar strategies can be used to insert heterologous DNA sequences at or between restriction sites in the cloned foreign gene.

Unfortunately, the range of insertion or deletion mutants that can be generated by the use of naturally occurring restriction sites is rather limited, and the endpoints of deletions (or the sites of insertion of foreign sequences) rarely fall exactly where the experimenter might wish. In these cases, the following three options are available:

1. Unique restriction sites can be created at defined locations by oligonucleotide-mediated mutagenesis. These sites can then be used to generate insertions and deletions by the methods discussed above.
2. A systematic method can be used to generate a large number of deletions or insertions within the general region of interest. These mutations can then be screened by fine-resolution restriction mapping and/or DNA sequencing for those whose termini happen to fall at desired locations. This strategy, although more laborious, is the method of choice when the target sites for insertion or deletion are not precisely known.
3. Specific segments of DNA can be deleted by oligonucleotide-mediated "loop-out" mutagenesis. This type of mutagenesis can also be used to insert short segments of synthetic DNA at defined locations ("loop-in" mutagenesis).

The generation of systematic deletions and insertions is discussed beginning on page 15.5. The generation of deletions and insertions by oligonucleotide-mediated mutagenesis is described beginning on page 15.51. These types of mutagenesis are used chiefly for two purposes:

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- To construct insertions and deletions within segments of DNA that carry out regulatory functions, for example, within the upstream elements that control expression of particular genes. The mutated versions of the regulatory elements are linked to an appropriate reporter gene whose level of expression can be quantitated in transfected cells. Experiments of this type often provide the first indication of the size and location of promoters and tissue-specific enhancers within regulatory regions.
- To truncate segments of cloned DNA as a prelude to DNA sequencing. The relative advantages of the various strategies that are used to generate and sequence deletion mutants are discussed in Chapter 13.

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SYSTEMATIC DELETIONS AND INSERTIONS

Linker-insertion Mutagenesis

Small deletions or insertions can be generated systematically in a number of different ways, two of which are described below. The first step in both procedures is the generation of a set of circularly permuted linear molecules by random double-stranded cleavage of superhelical plasmid DNA. The two methods differ in the techniques used to achieve this random cleavage.

1. *Partial digestion with pancreatic DNAase I.* Closed circular plasmid DNA is digested with limited amounts of DNAase I in the presence of a transition metal ion such as Mn^{++} or Co^{++} (Heffron et al. 1978). Under these conditions, DNAase I attacks DNA randomly, cutting both DNA strands at or near the same point to generate a population of circularly permuted linear molecules (Melgar and Goldthwait 1968). These unit-length linear molecules are purified to remove circular plasmid DNA, recircularized in the presence of a synthetic linker containing a restriction enzyme cleavage site that is not present in the plasmid DNA (Figure 15.1), and used to transform *E. coli*. The approximate positions of the synthetic linkers in plasmids carried by different transformants are then determined by restriction mapping. The chief disadvantages of this method are:

   - The entire plasmid is attacked during the first step (partial digestion with DNAase I). If the size of the target sequence is small compared with the size of the entire plasmid, only a small proportion of the mutants will map in the desired region. The impact of this problem can be minimized by using a vector that has been stripped of nonessential DNA sequences. Plasmids carrying linkers within the remaining essential sequences will not be able to transform *E. coli*.
   - The generation of linear DNA by partial digestion with DNAase I is inefficient and difficult to reproduce. The reaction is sensitive to the ionic strength of the buffer, to temperature, and to the degree of supercoiling of the template DNA (Campbell and Jackson 1980).
   - Linearization of plasmid DNA by random cleavage with DNAase I often creates ragged DNA termini that must be repaired by treatment with bacteriophage T4 DNA polymerase or the Klenow fragment of *E. coli* DNA polymerase I. Subsequent ligation of linkers to these termini often produces frameshifts and in-frame termination codons within protein-coding regions.
   - Clean separation of full-length linear DNA from supercoiled and relaxed plasmid DNA is difficult.

   Because of these difficulties, methods involving double-stranded cleavage of plasmid DNA with DNAase I are used only rarely. However, circumstances occasionally arise where no alternative procedure is available, for example, when large numbers of deletions are required and the number of naturally occurring restriction sites is limited. In these cases, use the procedure given on pages 15.27–15.31 for conversion of double-stranded plasmid DNA to linear DNA by digestion with DNAase I.

GENERATION OF LINKER-INSERTION MUTANTS WITH FREQUENTLY CUTTING RESTRICTION ENZYMES[1]

Before beginning mutagenesis, the following steps should be completed:

- The DNA to be mutagenized should be sequenced and cloned into a plasmid vector that contains as few nonessential sequences as possible.
- The sequence of the target DNA should be analyzed for the presence of suitable restriction sites. Enzyme(s) should be chosen that have potential cleavage sites distributed over the entire length of the target sequence. Often, it is necessary to use two or more restriction enzymes to obtain the desired type of distribution. Verify that any novel restriction site(s) created by insertion of the linker at these sites are not already present in the plasmid DNA.
- An aliquot of the preparation of recombinant plasmid DNA that is to be used as a substrate for mutagenesis should be analyzed by agarose gel electrophoresis. It should consist predominantly of superhelical molecules. If any linear plasmid is detectable, a new preparation should be made (see Chapter 1).

The major problem with this method is the generation of full-length linear molecules after partial digestion with a restriction enzyme that cleaves the recombinant plasmid DNA at many sites. Because most restriction enzymes work well on both linear and superhelical templates, there is no dependable way to prevent them from introducing supernumerary cleavages. A number of workers have used low concentrations of ethidium bromide to inhibit the activity of restriction enzymes on linear DNA (Österlund et al. 1982); others have used partial methylation of the superhelical template to reduce the total number of potential cleavage sites (see, e.g., Barany 1985b). In our experience, these methods are generally more trouble than they are worth. We recommend, instead, that conditions for optimal linearization of each preparation of plasmid DNA by particular batches of restriction enzyme be determined empirically as described below.

1. Set up a series of reactions that contain a constant amount of DNA and varying amounts of restriction enzyme:

   a. Prepare a tube containing 20 μg of superhelical plasmid DNA in a total volume of 100 μl of the appropriate 1 × restriction enzyme buffer. Dispense 19 μl of the mixture into one microfuge tube and 10 μl of the mixture into each of seven additional microfuge tubes. Store the tubes on ice.

   b. To the tube containing 19 μl of DNA solution, add 1 μl of restriction enzyme (3–15 units, depending on the enzyme and the manufacturer). Mix quickly, and replace the tube on ice.

[1]This method can be easily adapted for use with plasmid DNAs that have been linearized by digestion with pancreatic DNAase I (see pages 15.27–15.31). After the DNA has been purified and repaired by treatment with bacteriophage T4 DNA polymerase (steps 10–11, page 15.29), linkers should be added as described in steps 2–8 of this protocol. For a discussion of the difficulties of using DNAase I, see page 15.5.

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c. Using a fresh pipette tip, transfer 10 μl of the enzyme:DNA mixture to the second tube. Mix quickly, and replace the tube on ice.

d. Using another fresh pipette tip, transfer 10 μl from the second tube to the third tube. Continue this procedure until seven of the tubes contain both restriction enzyme and DNA. The eighth tube, which should not receive any restriction enzyme, is used as a control.

e. Transfer all eight tubes simultaneously from the ice to a water bath set at the appropriate temperature for the particular restriction enzyme. Incubate the reactions for 30 minutes.

f. While the tubes are incubating, digest approximately 1 μg of the plasmid DNA with an excess (5 units) of a restriction enzyme that cleaves the DNA only once. The resulting linear DNA will be used as an electrophoretic marker (see h).

g. After the eight test reactions have incubated for 30 minutes, quickly transfer the tubes to an ice-water bath. Add 5 μl of 50 mM EDTA (pH 8.0) to each tube to stop any further digestion by the enzyme.

h. Analyze 2 μl of each digest by electrophoresis through an agarose gel containing ethidium bromide (0.5 μg/ml), using as marker the plasmid DNA linearized in f. Electrophoresis should be continued until the linear DNA has migrated approximately one half the length of the gel.

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

i. Examine the gel by ultraviolet illumination. Identify the restriction digests that contain significant amounts of material that comigrates with the marker linear plasmid DNA.

Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

2. Pool the remainders of the restriction enzyme digests that contain significant amounts of full-length linear plasmid DNA. Isolate this material by electrophoresis through a preparative agarose gel, using as markers undigested superhelical plasmid DNA and plasmid DNA that has been cleaved to completion with a restriction enzyme that cleaves it only once. Recover the full-length linear DNA from the gel by one of the methods described in Chapter 6, aiming for a final concentration of ~100 ng/μl.

To minimize the possibility of contamination, at least two tracks of the gel should be left empty between the plasmid DNAs used as markers and the test samples.

3. Analyze an aliquot of the linearized DNA by gel electrophoresis, and estimate the total amount of DNA available for ligation. Calculate the amount of linkers that will be required for ligation to 100 ng of linearized plasmid DNA as follows:

Amount of linker (in nanograms) $= x/y \times z \times 100$

where x = length of linker (in nucleotides), y = length of linear plasmid DNA (in nucleotides), and z = desired molar ratio of linkers to target DNA.

Usually, a 20- to 30-fold molar excess of linkers is used to ensure that several copies of the linker are added to each end of the plasmid DNA before circularization occurs.

The synthetic linker *must* contain a restriction site that is not present in the original plasmid DNA.

4. Mix in the order given:

phosphorylated linkers	amount calculated above
linearized plasmid DNA	100 ng
H_2O to 18 μl	
10 × blunt-end ligation buffer	2 μl

The 10 × blunt-end ligation buffer should be added last to reduce the possibility that the DNA will be precipitated by spermidine.

Add 1–2 Weiss units of bacteriophage T4 DNA ligase, and incubate the reaction for 6–8 hours at 16°C.

10 × Blunt-end ligation buffer

0.66 M Tris · Cl (pH 7.6)
50 mM $MgCl_2$
50 mM dithiothreitol
1 mg/ml bovine serum albumin (Fraction V; Sigma) (optional)
10 mM hexamminecobalt chloride
2 mM ATP
5 mM spermidine HCl

Blunt-end ligation buffer should be stored in small aliquots at −20°C.

The spermidine and hexamminecobalt chloride [$(NH_3)_6CoCl_3$; Aldrich] combination increases the efficiency of ligation of linkers approximately fivefold.

Phosphorylated linkers containing recognition sequences for a large variety of restriction enzymes can be purchased from several manufacturers. Alternatively, bacteriophage T4 polynucleotide kinase can be used to add phosphate groups to the 5′ termini of unphosphorylated linkers, as described in step 2, page 15.63.

Usually, the ligation reaction goes well, and there is no need to check the products of the reaction before proceeding. If necessary, however, the ligation of linkers can be checked in two ways. During ligation, phosphorylated linkers should form dimers, trimers, tetramers, and longer oligomers that can be detected as a visible smear after an aliquot of the ligation mixture is analyzed by electrophoresis through a 1.8% agarose gel. Alternatively, a small amount of linkers that have been radiolabeled to high specific activity with [γ-^{32}P]ATP can be included in the ligation reaction. If ligation is successful, the radioactive linkers should form a series of radioactive bands that can be resolved by electrophoresis of the ligation products through a 15% denaturing polyacrylamide gel (see Chapter 13). These bands appear as a ladder on autoradiographs of the gel.

At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units (as defined by New England Biolabs). 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units.

5. Digest the linkers with the appropriate restriction enzyme(s) to remove polymers of linkers from the termini of the linearized plasmid DNA and to create cohesive termini. Either of two methods can be used to digest the linkers. In the first method, the sample is heated to inactivate DNA ligase and digested directly, without removal of the unincorporated linkers from the reaction. This method can be effective, but it requires comparatively large amounts of restriction enzyme(s) and will only work with restriction enzymes that are active in the ligation buffer. In the second method, excess linkers are removed by gel electrophoresis prior to digestion.

Method A

a. Inactivate the bacteriophage T4 DNA ligase by heating the ligation mixture to 68°C for 15 minutes.

b. Add a 50- to 100-fold excess of restriction enzyme (e.g., to a ligation reaction containing 100 ng of plasmid DNA, add 5–10 units of restriction enzyme). Incubate the reaction for 2 hours at the appropriate temperature.

c. Add EDTA to a final concentration of 5 mM, and purify the DNA by extraction with phenol:chloroform. Separate the organic and aqueous phases by centrifugation at 12,000*g* for 3 minutes at 4°C in a microfuge.

d. Carefully transfer the aqueous phase to the top of a spun column of Sephadex G-75. Separate the plasmid DNA from the remnants of linkers by spun-column chromatography as described in Appendix E.

e. To the eluate from the spun column, add 0.1 volume of 3 M sodium acetate (pH 5.2), and recover the plasmid DNA by precipitation with 2 volumes of ethanol for 30 minutes at 0°C.

f. Recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Remove the ethanol by aspiration, and allow the pellet of DNA to dry at room temperature. Redissolve the DNA in 20 μl of TE (pH 7.6).

g. Analyze an aliquot of the DNA (5 μl) by electrophoresis through a 0.8% agarose gel.

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Method B

a. Separate the linearized plasmid DNA from the linkers by electrophoresis through a 0.8% agarose gel. The plasmid DNA usually has a smeary appearance because of the heterogeneity in molecular weight caused by addition of variable numbers of linkers to its termini.

b. Recover the plasmid DNA from the gel by one of the methods described in Chapter 6.

c. Redissolve the plasmid DNA recovered from the gel in 20 μl of TE (pH 7.6).

d. Add 2.5 μl of the appropriate 10× restriction enzyme buffer and 2 units of the restriction enzyme that will digest the linkers added to the termini of the DNA.

e. Incubate the reaction for 2 hours at the appropriate temperature.

f. Add 80 μl of TE (pH 7.6), and extract the DNA with an equal volume of phenol:chloroform. Separate the organic and aqueous phases by centrifugation at 12,000*g* for 3 minutes at 4°C in a microfuge.

g. Transfer the aqueous phase to a fresh microfuge tube, and add:

10 M ammonium acetate	30 μl
isopropanol	120 μl

h. Store the mixture for 30 minutes at room temperature, and then recover the DNA by centrifugation at 12,000*g* for 10 minutes at room temperature in a microfuge. Remove the supernatant, wash the DNA pellet carefully with 70% ethanol, and recentrifuge.

i. Remove the ethanol by aspiration, and allow the pellet of DNA to dry at room temperature. Redissolve the DNA in 20 μl of TE (pH 7.6).

j. Analyze an aliquot of the DNA (5 μl) by electrophoresis through a 0.8% agarose gel. The DNA should no longer be smeary and should comigrate with full-length linear molecules of the original plasmid DNA.

6. To the remainder of the DNA, add:

TE (pH 7.6)	65 μl
10× ligase buffer	10 μl

Add 0.5 Weiss unit of bacteriophage T4 DNA ligase and 10 mM ATP to a final concentration of 1 mM. Incubate the reaction for 2–4 hours at 16°C.

10× Ligase buffer

0.2 M Tris · Cl (pH 7.6)
50 mM $MgCl_2$
50 mM dithiothreitol

This ligation reaction is carried out in a large volume to promote the formation of monomeric circular molecules and to reduce the formation of dimeric molecules.

7. Use aliquots of the ligation mixture to transform competent *E. coli* as described in Chapter 1, pages 1.74–1.83. Store the remainder of the ligation mixture at −20°C.

8. The next day, prepare minipreparations of plasmid DNA (see Chapter 1) from 12 independent transformants. Determine the approximate positions at which the linkers have been inserted by cleaving the plasmid DNAs with appropriate combinations of restriction enzymes, followed by gel electrophoresis.

   The best approach is to identify from the restriction map of the original plasmid two restriction enzymes that each cleave the plasmid once at different locations. Ideally, these two sites should flank the target DNA. In one tube, digest an aliquot of the test DNA with one of these enzymes *and* the restriction enzyme that cleaves the linker. In another tube, digest a second aliquot with the second restriction enzyme *and* the restriction enzyme that digests the linker. Examine the results of these digestions by agarose gel electrophoresis. If the test DNA carries a linker, each lane of the gel will contain two fragments of DNA whose sizes reflect the position of the linker relative to the two known restriction sites. From the analysis of the 12 independent transformants, estimate the number of independent transformants that will be required to saturate the original blunt-ended restriction sites in the target DNA with the desired density of linkers.

9. After preliminary restriction analysis of the transformants is completed, determine the exact position at which the linkers have been inserted in the target DNA by DNA sequencing (see Chapter 13). This is important because of the possibility that one or more of the enzymes used in the construction might have been contaminated, for example, with an exonuclease that might have removed a few nucleotides from either side of the site of insertion. In addition, DNA sequencing confirms that only one linker remains at the site of insertion.

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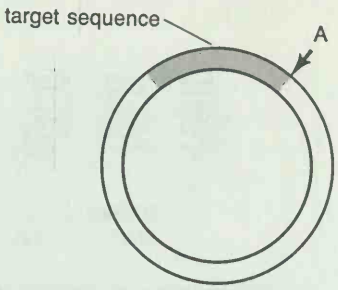
Generation of Nested Sets of Deletion Mutants

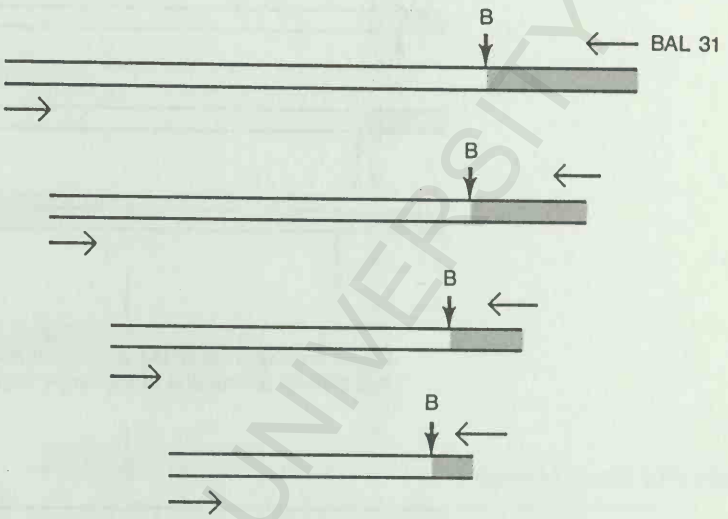
Several methods have been developed to generate nested sets of deletion mutants that lack progressively more sequences from one end or the other of the target DNA. These methods all rely on nucleases that digest DNA in a predictable fashion:

- *BAL 31:* This enzyme carries (1) an exonucleolytic activity that progressively degrades double-stranded linear DNA by liberating mononucleotides from the 3′ termini and (2) a weaker single-strand-specific endonucleolytic activity. Thus, the products of digestion of double-stranded DNA with BAL 31 are truncated molecules carrying blunt ends or short protruding 5′ termini. To generate deletion mutants, recombinant plasmid, phagemid, or bacteriophage M13 replicative form DNA is linearized with a restriction enzyme that cleaves at one end of the target sequence. The double-stranded linear DNA is digested with BAL 31 for different lengths of time, the termini are repaired, and the truncated fragments of target DNA are then cleaved from the remainder of the vector with a second restriction enzyme and recloned (see Figure 15.2).
- *Pancreatic DNAase I:* In the presence of Mg^{++}, DNAase I behaves like an endonuclease that introduces nicks independently into each strand of double-stranded DNA. However, as discussed earlier, in the presence of a transition metal ion such as Mn^{++} or Co^{++}, the enzyme cuts both strands of DNA at approximately the same place (Melgar and Goldthwait 1968). This property can be used to create deletions in a segment of target DNA that has been cloned in a plasmid, phagemid, or bacteriophage M13 vector (Hong 1982, 1987; Labeit et al. 1987). Because the sites of cleavage are distributed in a statistically random fashion, a fraction of the original population of closed circular DNAs is converted by DNAase I into a permuted set of linear molecules. These are then isolated, repaired, and digested with a restriction enzyme whose unique site of cleavage lies at one end of the target DNA. Recircularization of the resulting population therefore generates clones that lack sequences lying between the site of DNAase I cleavage and the restriction site (see Figure 15.3).
- *Exonuclease III:* This enzyme catalyzes the stepwise removal of 5′ mononucleotides from the recessed or blunt 3′ hydroxyl termini of double-stranded DNA (Weiss 1976). However, protruding 3′ termini are completely resistant to the activity of the enzyme (Rogers and Weiss 1980). To create deletion mutants (Guo and Wu 1983; Henikoff 1984, 1987), the double-stranded DNA of recombinant plasmid, phagemid, or bacteriophage M13 replicative form DNA is digested with two restriction enzymes whose sites of cleavage both lie between one end of the target DNA and the binding site for universal sequencing primer. The enzyme that cleaves nearer the target sequence must generate either a blunt end or a recessed 3′ terminus; the other enzyme must generate a 4-nucleotide protruding 3′ terminus (see Figure 15.4). Because only the blunt or recessed 3′ terminus of the resulting linear DNA is susceptible to exonuclease III, digestion proceeds unidirectionally away from the site of cleavage into the target DNA sequence. The exposed single strands are then removed by digestion with

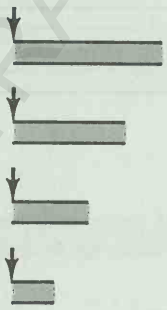


Digest plasmid DNA with a restriction enzyme (A) that cleaves at one end of the target sequence.

Digest linear DNA with BAL 31 for different lengths of time



Repair termini of target fragments with DNA polymerases.
Recover truncated target sequence by digestion with a restriction enzyme (B) that cleaves at the opposite end of the target sequence.



Clone fragments in a plasmid, phagemid, or bacteriophage M13 vector.

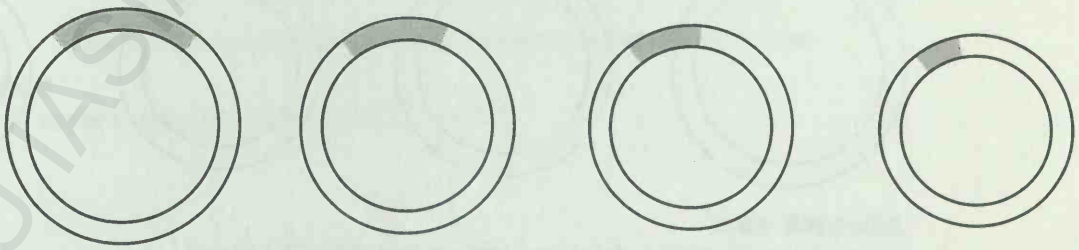


FIGURE 15.2
Generation of nested deletions with BAL 31.

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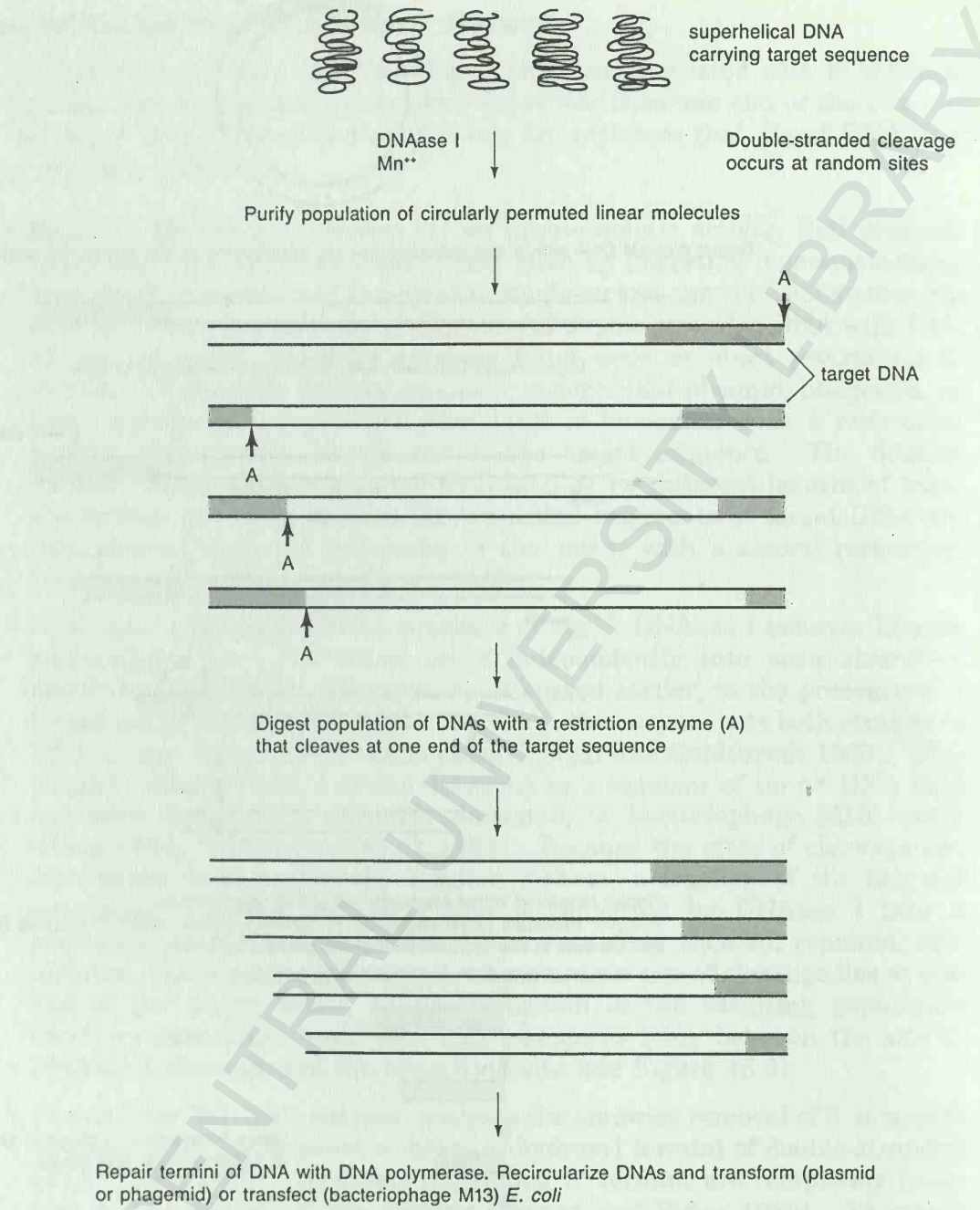
superhelical DNA
carrying target sequence
DNAase I
Mn++
Double-stranded cleavage
occurs at random sites
Purify population of circularly permuted linear molecules
A
target DNA
A
A
A
Digest population of DNAs with a restriction enzyme (A)
that cleaves at one end of the target sequence
Repair termini of DNA with DNA polymerase. Recircularize DNAs and transform (plasmid
or phagemid) or transfect (bacteriophage M13) E. coli

FIGURE 15.3
Generation of nested deletions with pancreatic DNAase I.

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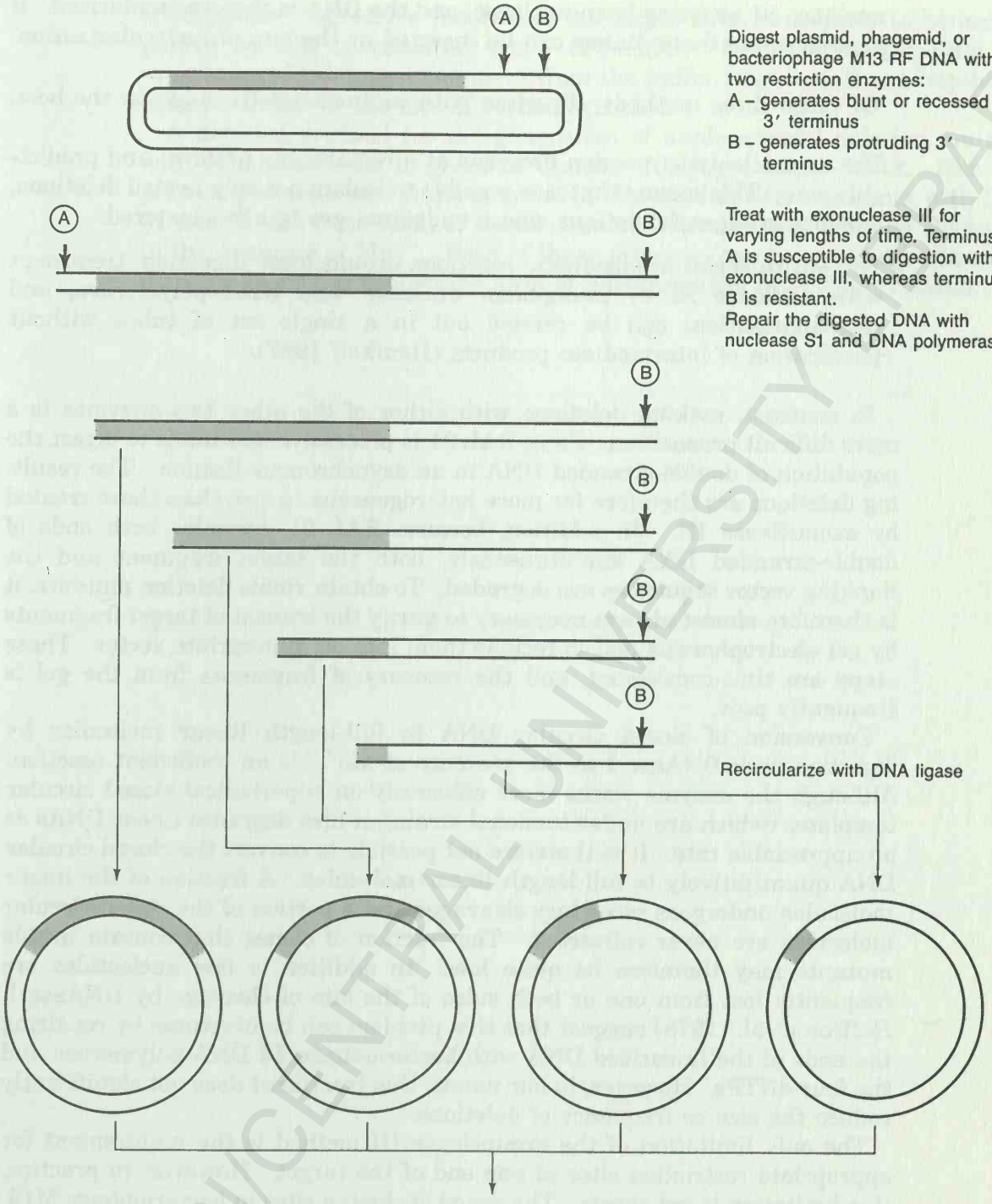
A
B
Digest plasmid, phagemid, or bacteriophage M13 RF DNA with two restriction enzymes:
A – generates blunt or recessed 3′ terminus
B – generates protruding 3′ terminus
Treat with exonuclease III for varying lengths of time. Terminus A is susceptible to digestion with exonuclease III, whereas terminus B is resistant.
Repair the digested DNA with nuclease S1 and DNA polymerase.
Recircularize with DNA ligase
Transform (plasmids and phagemids) or transfect (bacteriophage M13) E. coli

FIGURE 15.4
Generation of nested deletions with exonuclease III.

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nuclease S1 or mung-bean nuclease, and the DNA is then recircularized. If desired, a synthetic linker can be inserted at the site of recircularization.

Of these three methods, digestion with exonuclease III is by far the best:

- The exonucleolytic reaction proceeds at a remarkably uniform and predictable rate. This means that it is possible to isolate not only nested deletions, but also groups of deletions whose endpoints are tightly clustered.
- The entire series of enzymatic reactions (exonuclease digestion, treatment with nuclease S1 or mung-bean nuclease and DNA polymerase, and recircularization) can be carried out in a single set of tubes without purification of intermediate products (Henikoff 1987).

In contrast, making deletions with either of the other two enzymes is a more difficult proposition. First, BAL 31 is processive and tends to digest the population of double-stranded DNA in an asynchronous fashion. The resulting deletions are therefore far more heterogeneous in size than those created by exonuclease III. In addition, because BAL 31 degrades both ends of double-stranded DNA simultaneously, both the target fragment and the flanking vector sequences are degraded. To obtain viable deletion mutants, it is therefore almost always necessary to purify the truncated target fragments by gel electrophoresis and to reclone them into an appropriate vector. These steps are time-consuming, and the recovery of fragments from the gel is frequently poor.

Conversion of closed circular DNA to full-length linear molecules by digestion with DNAase I in the presence of Mn^{++} is an inefficient reaction. Although the enzyme works more efficiently on superhelical closed circular templates (which are under torsional strain), it also degrades linear DNAs at an appreciable rate. It is therefore not possible to convert the closed circular DNA quantitatively to full-length linear molecules. A fraction of the linear molecules undergoes secondary cleavages and a portion of the closed circular molecules are never converted. The fraction of clones that contain usable mutants may therefore be quite low. In addition, a few nucleotides are frequently lost from one or both sides of the site of cleavage by DNAase I. Heffron et al. (1978) suggest that this problem can be overcome by repairing the ends of the linearized DNA with bacteriophage T4 DNA polymerase and the four dNTPs. However, in our hands, this treatment does not significantly reduce the size or frequency of deletions.

The only limitation of the exonuclease III method is the requirement for appropriate restriction sites at one end of the target. However, in practice, this limitation is not severe. The range of cloning sites in bacteriophage M13, phagemid, and plasmid vectors is now so large that it is almost always possible to find a vector that is suitable for the particular target fragment under study. If necessary, recessed 3′ termini can be protected from attack by filling with the Klenow fragment of *E. coli* DNA polymerase I and α-thiophosphate dNTPs (Jasin et al. 1983). Alternatively, a "buffer" fragment can be inserted between the site of cleavage and the flanking vector sequences (Haltiner et al. 1985). As long as the buffer fragment is long

enough, the progressive removal of the target DNA is unlikely to be accompanied by loss of essential vector sequences. However, to facilitate the separation of the target fragment from the buffer fragment, it is advisable to insert a linker at the site of recircularization.

A detailed protocol for the generation of unidirectional deletion mutants with exonuclease III is given in Chapter 13, pages 13.39–13.41. Here we present protocols for (1) the generation of nested deletion mutants with BAL 31 and (2) the conversion of closed circular DNA to linear DNA by DNAase I in the presence of Mn^{++}. Both of these protocols can be used with plasmids, phagemids, or the replicative form of bacteriophage M13 recombinants.

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The rate at which BAL 31 removes nucleotides from the termini of double-stranded DNA is given by the equation:

$$\frac{dM_t}{dt} = \frac{-2V_m M_n}{[K_m + (S)_o]}$$

where M_t = molecular weight of linear double-stranded DNA after t minutes of digestion, V_m = maximum reaction velocity (expressed as moles of nucleotide removed/liter/minute), M_n = average molecular mass of sodium mononucleotide (taken as 330 daltons), K_m = Michaelis-Menten constant (expressed as moles of double-stranded DNA termini/liter, and $(S)_o$ = molar concentration of double-stranded termini/liter at $t = 0$ minutes (remains essentially constant until a significant portion of the molecules has undergone complete degradation).

At an enzyme concentration of 40 units/ml:

$V_m = 2.4 \times 10^{-5}$ moles of nucleotide removed/liter/minute
$K_m = 4.9 \times 10^{-9}$ moles of double-stranded DNA termini/liter

Assuming that the value of V_m alters in proportion to enzyme dilution (5/40 in the example below where the enzyme concentration is 5 units/ml), then the rate of degradation of a 2-kb fragment of DNA in a 100-μl reaction containing 10 μg of DNA and 0.5 unit of enzyme will be:

$$= \frac{-2(5/40)(2.4 \times 10^{-5})(330)}{(4.9 \times 10^{-9}) + (1.5 \times 10^{-7})}$$

$$= \frac{-(1/4)(2.4 \times 10^{-5})(330)}{1.5049 \times 10^{-7}}$$

$= -1.31 \times 10^{-4}$ daltons/minute/DNA molecule

$= -19$ bp/minute/DNA molecule

$= -9.5$ bp/minute/terminus of double-stranded DNA

In most cases (i.e., when digesting DNAs less than 10 kb in length and when the concentration of DNA in the reaction exceeds 20 μg/ml), the term for K_m in the denominator of the equation becomes insignificant.

The 3′ exonuclease activity of BAL 31 is about 20-fold more efficient than that of the single-strand-specific endonuclease. Thus, the average length of single-stranded tails created by digestion of linear double-stranded DNA is dependent on the enzyme concentration. At high concentrations (2–5 units/ml), an average of 5 nucleotides of single-stranded DNA remain per terminus, and approximately 10–20% of the molecules can be ligated to blunt-ended DNA without further treatment. At low enzyme concentrations (0.1–0.2 units/ml), the single-stranded termini may be very long and the efficiency of blunt-end ligation is very low. Whatever the concentration of enzyme used, repair of the protruding termini with bacteriophage T4 DNA polymerase (and, in some cases, the Klenow fragment of *E. coli* DNA polymerase I) greatly increases the efficiency with which deletion mutants can be recovered.

Preparation of target DNA for digestion by BAL 31

1. Clone the target fragment into an appropriate plasmid or bacteriophage M13 vector. If deletion mutants are to be constructed from both termini of the target DNA, it will be necessary to clone the parental target DNA in both orientations with respect to the polycloning site in vectors such as pUC18 and pUC19, pSP64 and pSP65, bacteriophage M13mp18 and M13mp19, etc.

2. Purify the closed circular recombinant DNA(s) by equilibrium centrifugation in CsCl–ethidium bromide gradients (see Chapter 1, pages 1.42–1.44).

   It is essential to use highly purified closed circular DNA for two reasons: (1) to minimize the contribution of contaminating RNA and small fragments of *E. coli* chromosomal DNA to the total concentration of termini in the reaction and (2) to eliminate nicked circular molecules, which are degraded by BAL 31 from the site of the nick.

3. Cleave 30 μg of the closed circular DNAs to completion with a restriction enzyme that cleaves at one end of the target DNA. This site defines the common point from which the nested deletions will begin. Use agarose gel electrophoresis to verify that digestion with the restriction enzyme is complete.

4. Purify the DNA by extraction with an equal volume of phenol:chloroform. Separate the aqueous and organic phases by centrifugation at 12,000*g* for 3 minutes at 0°C in a microfuge, and then transfer the aqueous phase to a fresh microfuge tube. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol at 0°C. Store the tube for 10 minutes at 0°C, and then recover the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Remove the supernatant, and wash the pellet of DNA carefully with 70% ethanol at room temperature. Dry the pellet at room temperature, and then redissolve it in TE (pH 7.6) at a concentration of 1 μg/μl. Store the DNA at −20°C.

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Trial reactions with BAL 31

Most commercial preparations of BAL 31 contain two kinetically distinct forms of the enzyme: a fast form and a slow form. The slow form is a proteolytic degradation product of the fast form. The rate at which BAL 31 digests DNA is a function of the proportion of the fast and slow forms in the particular preparation of the enzyme used. Pure preparations of the fast form are available (Wei et al. 1983), but they are expensive and, in any case, they frequently decay into the slow form during storage. To preserve BAL 31 in the fast form, the enzyme should not be frozen but should be stored at 4°C.

Because the ratio of fast and slow forms of BAL 31 varies from preparation to preparation, it is essential to assay, by the following procedures, the activity of the particular batch of enzyme that will be used to generate deletions.

1. Mix:

linearized DNA (1 μg/μl)	4 μl
H_2O	48 μl
5 × BAL 31 buffer	13 μl

Dispense 9 μl of this mixture into each of seven separate microfuge tubes.

5 × BAL 31 buffer

3 M NaCl
60 mM $CaCl_2$
60 mM $MgCl_2$
100 mM Tris · Cl (pH 8.0)
1 mM EDTA (pH 8.0)

2. Make a series of seven twofold dilutions of BAL 31 in 1 × BAL 31 buffer. This is best carried out by placing seven drops of 1 × BAL 31 buffer (2 μl each) on the surface of a piece of Parafilm lying on a bed of ice. Using a disposable micropipette tip, mix 2 μl of the BAL 31 preparation being tested with the first drop. Using a fresh tip, transfer 2 μl of the mixture to the next drop, and again mix. Continue in this fashion until enzyme has been added to all of the drops. Working quickly, add 1 μl of each of the last six dilutions to six of the microfuge tubes containing the linear DNA being tested. Do not add enzyme to the seventh tube.

3. Incubate all of the microfuge tubes (including the tube that received no enzyme) for 30 minutes at 37°C.

4. Add 1 μl of 200 mM EGTA (pH 8.0) to each tube, and then heat the tubes to 65°C for 5 minutes.

BAL 31 requires Ca^{++} for activity and is therefore completely inhibited by EGTA. The enzyme is also inactivated by heating to 65°C.

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5. Mix each of the samples with 3 μl of agarose gel electrophoresis buffer (e.g., 0.5 × TBE; see Appendix B), and analyze the size of the DNAs by electrophoresis through a 0.8% agarose gel containing ethidium bromide (0.5 μg/ml).

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Follow precautions detailed in step 1h, page 15.9.

6. Examine the gel by ultraviolet illumination, and determine the dilution of enzyme that is just sufficient to digest the DNA to the point where only a smear of small (200-bp) fragments is detectable. This dilution of BAL 31 will be used in the large-scale digestion (step 3, page 15.24).

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. Follow precautions detailed in step 1i, page 15.9.

   Most commercial preparations of BAL 31 are supplied at a concentration of approximately 1 unit/μl; 0.05–0.1 unit of most commercial preparations of BAL 31 is sufficient to digest 1 μg of a 2-kb segment of linear DNA to fragments $<$200 bp in length.

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Generation of nested deletions with BAL 31

1. Mix:

linearized DNA (1 μg/μl)	20 μl
H_2O	240 μl
5× BAL 31 buffer (see page 15.22)	65 μl

Incubate the mixture in a water bath set at 37°C.

2. While the mixture is warming to 37°C, prepare a set of eight microfuge tubes, each containing 5 μl of 200 mM EGTA (pH 8.0). Label the tubes 1.5 minutes, 3.0 minutes, 4.5 minutes, etc.

3. Add 36 μl of the appropriate dilution of BAL 31 (see step 6, page 15.23) to the reaction mixture prepared in step 1. Quickly mix the enzyme by tapping the side of the tube, and then return the tube to the water bath set at 37°C and start a stopwatch.

4. At 1.5-minute intervals, transfer 45 μl of the reaction mixture to the appropriately labeled microfuge tube. Store the tubes on ice until all of the samples have been collected.

5. Heat the tubes to 65°C for 5 minutes to inactivate the BAL 31.

6. Add 5 μl of 3 M sodium acetate (pH 5.2) to each tube, followed by 100 μl of ice-cold ethanol. Mix the solution by vortexing, and store the tubes on ice for 20–30 minutes.

7. Recover the DNAs by centrifugation at 12,000*g* for 10 minutes at 4°C. Carefully remove the supernatants, and wash the pellets with 200 μl of ice-cold 70% ethanol. Centrifuge for a further 2 minutes.

8. Carefully remove the supernatants, and stand the open tubes at room temperature until all of the ethanol has evaporated.

9. Dissolve each of the pellets in 23 μl of TE (pH 7.6).

10. Add to each of the DNA preparations:

a solution containing all four dNTPs, each at a concentration of 0.5 mM	3 μl
10× polymerase buffer	3 μl
bacteriophage T4 DNA polymerase (~5 units)	1 μl

Incubate the reactions for 15 minutes at room temperature, and then add approximately 1 μl (~5 units) of the Klenow fragment of *E. coli* DNA polymerase I. Continue the incubation for a further 15 minutes at room temperature.

The use of two different DNA polymerases in the repair reaction results in an approximately threefold increase in recovery of mutants.

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10 × Polymerase buffer

200 mM Tris · Cl (pH 7.6)
10 mM $MgCl_2$
1 mM dithiothreitol

11. Purify the DNAs by extraction with phenol:chloroform, and then precipitate the DNAs with ethanol as described in steps 6–8. Redissolve each of the DNAs in 16 μl of TE (pH 7.6).

12. To each DNA, add 2 μl of the appropriate 10 × restriction enzyme buffer and 8 units of a restriction enzyme that will separate the target DNA from the vector. Incubate the reactions for 1 hour at the appropriate temperature.

13. At the end of the incubation, transfer an aliquot (3 μl) from each digest to a fresh microfuge tube. Store the remainders of the digests on ice until needed in step 15. Add 1 μl of gel-loading buffer (see Appendix B) to each aliquot, and load the contents of each tube into the wells of an agarose gel containing ethidium bromide (0.5 μg/ml). The wells at the sides of the gel should contain markers of the appropriate size.

    Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Follow precautions detailed in step 1h, page 15.9.

    The gel should be cast with a percentage of agarose that allows separation of the target fragment and the vector (see Chapter 6, page 6.5).

14. Separate the target fragments and vector DNA by electrophoresis. Examine the gel by ultraviolet illumination, and determine which of the samples has been digested to an appropriate size by BAL 31.

    Caution: Ultraviolet radiation is dangerous, particularly to the eyes. Follow precautions detailed in step 1i, page 15.9.

15. Pool the samples containing target DNA of the appropriate size, and purify the target fragments by preparative gel electrophoresis as described in Chapter 6.

16. Estimate the amount of purified target DNA from the intensity of ethidium-bromide-mediated fluorescence (see Appendix E).

17. Ligate the repaired DNA with a plasmid, phagemid, or bacteriophage M13 vector (see Chapters 1 and 4) that carries one blunt end and one terminus that is compatible with the restriction enzyme used in step 12. To reduce the background of colonies carrying nonrecombinants, the vector should be dephosphorylated with calf intestinal alkaline phosphatase as described in Appendix F.

    The exact composition and volume of the ligation reaction will depend on the amount of target DNA available. If possible, use between 50 and 100 ng of target DNA, and

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make sure that the molar ratio of vector DNA to target DNA is at least 5 (to minimize the number of recombinants that contain more than one fragment of target DNA). To maximize the formation of recombinants, the ligation reaction should be carried out under conditions that favor blunt-end ligation, i.e., in a small volume in the presence of high concentrations of bacteriophage T4 DNA ligase and low concentrations of ATP.

18. Transform (plasmids or phagemids) or transfect (bacteriophage M13 replicative form DNA) competent *E. coli* of an appropriate strain with 1-μl and 5-μl aliquots of the ligation mixture. The next day, grow small-scale cultures of 12 transformed colonies or bacteriophage M13 plaques, chosen at random.

19. Purify plasmid, phagemid, or bacteriophage M13 replicative form DNA from each of the 12 cultures by using one of the methods described in Chapters 1 and 4. Digest the DNAs with a restriction enzyme(s) that will liberate the target fragment from the vector.

20. Analyze the size of the target fragment liberated from each of the DNAs by agarose gel electrophoresis, using markers of an appropriate size.

21. If the results are satisfactory (i.e., if the target fragments fall within the desired size range), pick a large number of individual transformed colonies or plaques and determine the size of the inserts as described above. Preserve those cultures that carry recombinants of the desired size.

22. Determine the exact endpoints of the deletion in each mutant by DNA sequencing (see Chapter 13).

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CLEAVAGE OF DOUBLE-STRANDED CLOSED CIRCULAR DNA WITH PANCREATIC DNAase I IN THE PRESENCE OF Mn^{++}

The following protocol is an adaptation of methods described by Anderson (1981) and Labeit et al. (1987). The principle of the method is shown in Figure 15.3. In brief, a double-stranded closed circular DNA (plasmid, phagemid, or bacteriophage M13 replicative form DNA) carrying the target DNA is digested with limiting concentrations of pancreatic DNAase I in the presence of Mn^{++}. Under these conditions, the nuclease cleaves essentially at random and converts a small fraction of the DNA to linear molecules. Few of the molecules are cleaved more than once by the enzyme. Digestion with a restriction enzyme that cleaves at one end of the target sequence therefore generates a mixed population that contains full-length linear molecules (derived from the closed circles that escaped digestion with pancreatic DNAase I) and deleted molecules (derived from the molecules that were cleaved by pancreatic DNAase I). The termini of the linear molecules are then repaired with bacteriophage T4 DNA polymerase, the Klenow fragment of *E. coli* DNA polymerase I, and the four dNTPs and ligated to generate a new population of closed circular molecules. If possible, the DNA is digested with a second restriction enzyme that cleaves somewhere within the region targeted for deletion to eliminate the members of this population that escaped deletion. Because closed circular molecules transform (or transfect) *E. coli* much more efficiently than linear molecules, most of the resulting colonies or plaques will be generated by recombinants that have undergone deletion.

1. Make a stock solution of DNAase I (1 mg/ml in 0.01 N HCl). Divide the enzyme solution into small aliquots (10 μl), and store them in small microfuge tubes at −70°C.

2. When you are ready to start the digestion, remove an aliquot of DNAase I from storage at −70°C and dilute it 1:1000 into ice-cold 1 × DNAase/Mn^{++} digestion buffer. The concentration of the diluted enzyme should be 10 ng/μl.

10 × DNAase/Mn^{++} digestion buffer

500 mM Tris · Cl (pH 7.6)
100 mM $MnCl_2$
1 mg/ml bovine serum albumin (Fraction V; Sigma)

3. In a microfuge tube, mix:

closed circular DNA 5–10 μg
H_2O to 50 μl

Store the microfuge tube at room temperature.

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Minipreparations of plasmid or bacteriophage M13 replicative form DNA extracted from bacterial cells by any of the methods described in Chapters 1 and 4, respectively, work adequately in this method. However, the most reproducible results are obtained with closed circular DNA that has been purified by equilibrium centrifugation in CsCl–ethidium bromide gradients (see Chapter 1, page 1.42–1.44).

4. In a separate microfuge tube, mix:

Tris · Cl (pH 7.4)	80 μl
1 M $MnCl_2$	2 μl
H_2O	908 μl
10^3-fold dilution of stock DNAase I (see step 2)	5 μl

5. Immediately add 50 μl of the contents of this microfuge tube to the tube containing the closed circular DNA. Start a stopwatch, and incubate the digestion mixture at room temperature.

6. At 1.5-minute intervals, withdraw 18-μl samples of the digestion mixture and place in microfuge tubes that contain 2 μl of 0.1 M EDTA (pH 8.0). Store the collected samples on ice.

7. When all of the samples have been collected, analyze 3 μl of each sample by electrophoresis through an agarose gel cast and run at <1 V/cm in 0.5 × TBE (see Appendix B) containing ethidium bromide (0.5 μg/ml). Under these conditions, closed circular DNA migrates most rapidly through the gel, followed by linear DNA. Relaxed circular DNA runs a distant third. As markers, use an aliquot of the original closed circular DNA and an aliquot of the same DNA that has been digested to completion with a restriction enzyme that cleaves the DNA once.

   Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Follow precautions detailed in step 1h, page 15.9.

   While the gel is running, store the remainders of the samples on ice.

8. Examine the gel by ultraviolet illumination, and pool the samples in which approximately 20–33% of the closed circular DNA has been converted to the linear form. Adjust the volume to 100 μl with TE (pH 7.6), and then extract the pooled sample once with an equal volume of phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube, and add 0.1 volume of 3 M sodium acetate (pH 5.2). Precipitate the DNA with 2 volumes of ethanol for 15 minutes at 0°C.

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. Follow precautions detailed in step 1i, page 15.9.

9. Collect the precipitated DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Remove the supernatant, and wash the pellet carefully in 70% ethanol at room temperature. Allow the pellet to dry at room temperature, and then redissolve it in 16 μl of TE (pH 7.6). Add 2 μl of the appropriate 10 × restriction enzyme buffer and 5–10 units of a restriction enzyme that cleaves in a nonessential region of the vector upstream of the sequences to be deleted. Incubate the reaction mixture for 2 hours at the appropriate temperature.

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10. Add:

a solution containing all four dNTPs, each at a concentration of 0.5 mM	3 μl
10 × polymerase buffer	3 μl
bacteriophage T4 DNA polymerase (~5 units)	1 μl

Incubate the reactions for 15 minutes at room temperature, and then add approximately 1 μl (~5 units) of the Klenow fragment of *E. coli* DNA polymerase I. Continue the incubation for a further 15 minutes at room temperature.

10 × Polymerase buffer

200 mM Tris · Cl (pH 7.6)
10 mM $MgCl_2$
1 mM dithiothreitol

The use of two different DNA polymerases in the repair reaction results in an approximately threefold increase in recovery of mutants.

11. Add 70 μl of TE (pH 7.6), and then extract the sample once with an equal volume of phenol:chloroform. Transfer the aqueous phase to a fresh microfuge tube, and add 0.1 volume of 3 M sodium acetate (pH 5.2). Precipitate the DNA with 2 volumes of ethanol for 15 minutes at 0°C.

12. Collect the precipitated DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Remove the supernatant, and wash the pellet carefully in 70% ethanol at room temperature. Allow the pellet to dry at room temperature, and then redissolve it in 17 μl of TE (pH 7.6).

13. Recircularize the deleted molecules by adding 2 μl of 10 × blunt-end ligation buffer and approximately 1 Weiss unit of bacteriophage T4 DNA ligase. Incubate the ligation mixture for 12 hours at 16°C.

10 × Blunt-end ligation buffer

0.66 M Tris · Cl (pH 7.6)
50 mM $MgCl_2$
50 mM dithiothreitol
1 mg/ml bovine serum albumin (Fraction V; Sigma) (optional)
10 mM hexamminecobalt chloride
2 mM ATP
5 mM spermidine HCl

Blunt-end ligation buffer should be stored in small aliquots at −20°C.

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To prevent the formation of concatemers, it is important that the concentration of termini in the ligation reaction should not exceed 5–6 nM. This concentration is equivalent to 10 μg/ml of a plasmid of the size of pBR322.

If desired, a phosphorylated linker can be inserted at the site of recircularization by including the linker during ligation. Protocols are given on page 15.10 of this chapter and in Appendix F.

At least three different assays are used to measure the activity of bacteriophage T4 DNA ligase. Most manufacturers (apart from New England Biolabs) now calibrate the enzyme in Weiss units (Weiss et al. 1968). One Weiss unit is the amount of enzyme that catalyzes the exchange of 1 nmole of ^{32}P from pyrophosphate into [γ,β-^{32}P]ATP in 20 minutes at 37°C. One Weiss unit corresponds to 0.2 unit determined in the exonuclease resistance assay (Modrich and Lehman 1970) and to 60 cohesive-end units (as defined by New England Biolabs). 0.015 Weiss unit of bacteriophage T4 DNA ligase therefore will ligate 50% of the *Hin*dIII fragments of bacteriophage λ (5 μg) in 30 minutes at 16°C. Throughout this manual, bacteriophage T4 DNA ligase is given in Weiss units.

14. Heat the ligation reaction to 68°C for 10 minutes to inactivate the bacteriophage T4 DNA ligase.

15. (*Optional*) If the region targeted for deletion contains a restriction enzyme cleavage site not found elsewhere in the original DNA, digest the ligated DNA with the restriction enzyme as follows:

Allow the ligation reaction to cool to room temperature, and then add:

TE (pH 7.6)	22 μl
the appropriate 10× restriction enzyme buffer	5 μl
the appropriate restriction enzyme	5–10 units

Incubate the reaction for 2 hours at the appropriate temperature.

16. Transform (plasmids or phagemids) or transfect (bacteriophage M13 replicative form DNA) competent *E. coli* of the appropriate strain (see Chapters 1 and 4, respectively) with 10 μl of the restriction digest.

17. The next day, grow small-scale cultures of 12 colonies or plaques, chosen at random.

18. Purify plasmid, phagemid, or bacteriophage M13 replicative form DNA from each of the 12 cultures (see Chapters 1 and 4). Digest the DNAs with a restriction enzyme(s) that will liberate the target fragment from the vector.

19. Analyze the size of the target fragment liberated from each of the plasmids by agarose gel electrophoresis, using markers of an appropriate size.

20. If the results are satisfactory (i.e., if the target fragments fall within the desired size range), pick a large number of individual transformed colonies or plaques and determine the size of the inserts as described above. Preserve those cultures that carry recombinants of the desired size.

21. Determine the exact endpoints of the deletion in each mutant by DNA sequencing (see Chapter 13).

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Linker-scanning Mutagenesis

The identification and localization of regulatory regions in DNA usually begins with the analysis of nested sets of deletions that penetrate into the region of interest from the upstream and downstream directions. Such mutants, which can be generated by techniques described elsewhere in this chapter, allow the outer borders of the regulatory domain to be defined and open the way to more precise mapping and analysis of internal subdomains. In many cases, however, the region defined by nested sets of deletion mutants remains too large to be immediately accessible to analysis by substitution of individual bases. Under such circumstances, it is best to use another form of mutagenesis to search within the regulatory region for sequences of particular importance. This can be achieved by constructing "linker-scanning" mutations (McKnight and Kingsbury 1982). As shown in Figure 15.5, such mutations are generated by ligating 5′ and 3′ deletion mutants whose endpoints differ by precisely the number of nucleotides present in the synthetic DNA linker. When these deletions are joined via the linker, the nucleotides ordinarily present in the intact DNA sequence will be replaced by the linker sequence.

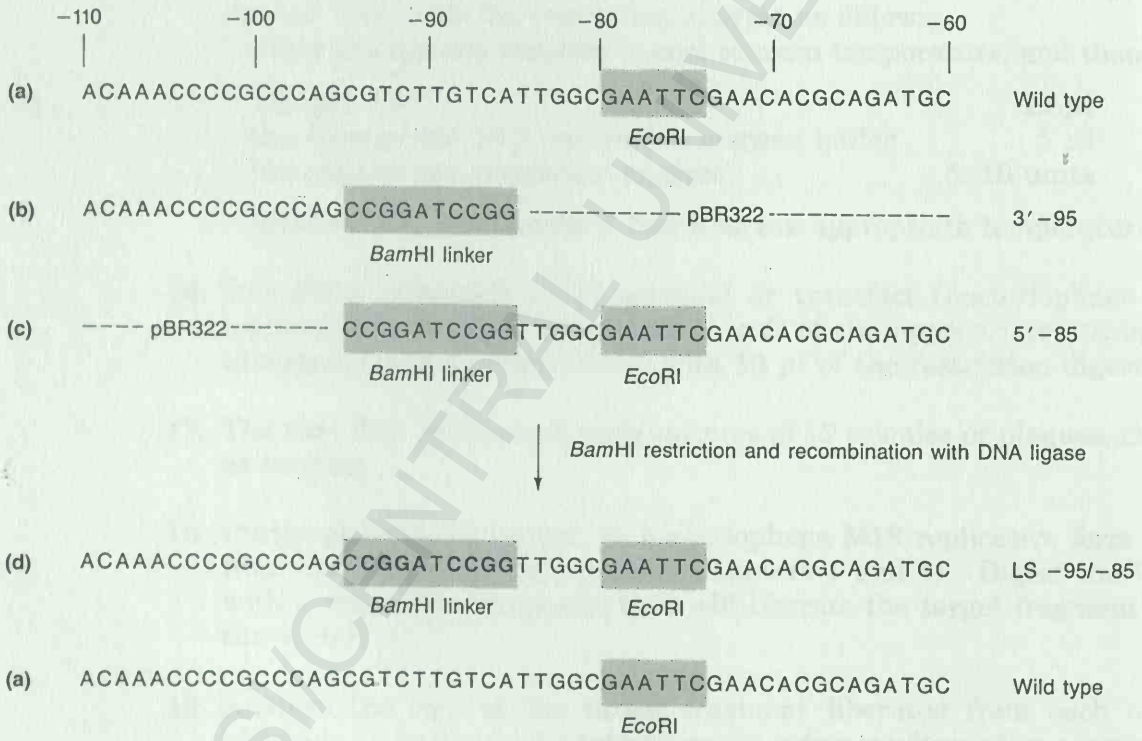


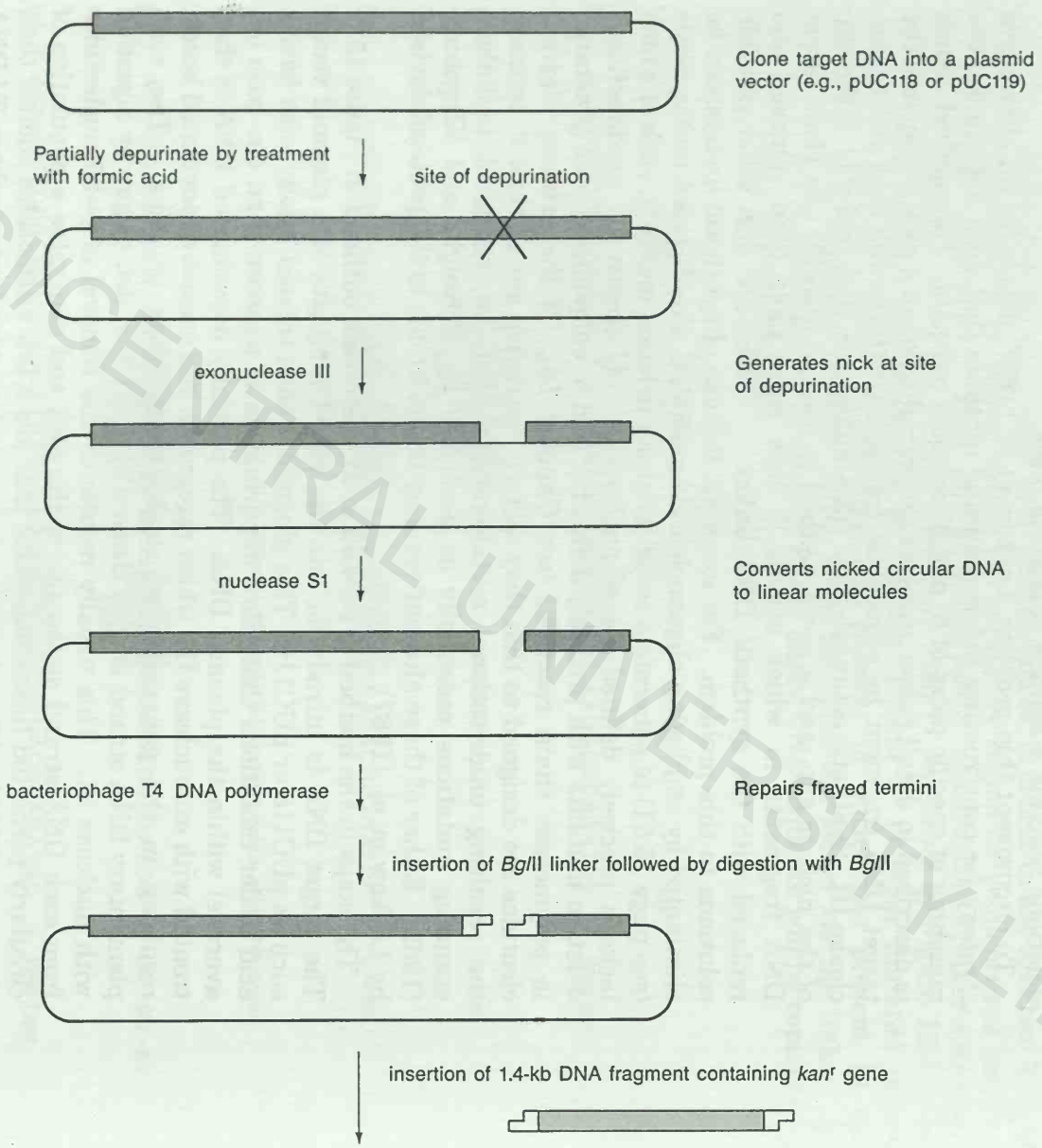
FIGURE 15.5
Construction of a linker-scanning mutant. (*a*) The nucleotide sequence of the normal HSV *tk* gene between 60 and 110 residues upstream of the transcription initiation site is shown on the top and bottom lines. This sequence is referred to as wild type. (*b, c*) The nucleotide sequences of two linker deletion mutants 3′ − 95 and 5′ − 85. (*d*) The linker-scanning mutant −95/−85 was constructed by recombining 3′ − 95 with 5′ − 85 at the *Bam*HI restriction site. The nucleotide residues of the *tk* gene that are substituted by this procedure are shown in bold type. (Redrawn, with permission, from McKnight and Kingsbury 1982, *Science,* v. 217, p. 318. © 1982 by the AAAS.)

In practice, the construction of a complete set of linker-scanning mutations for a stretch of sequence as short as 100 bp is a laborious undertaking. To obtain a usable set of linker-scanning mutants, a large number of 5′ and 3′ deletions must be constructed, sequenced, and matched. For example, in the original description of the method (McKnight and Kingsbury 1982), 43 5′ deletions and 42 3′ deletions were constructed to obtain 15 perfectly matched pairs of linker-scanning mutants. Although the original method for constructing such mutants was later streamlined (Haltiner et al. 1985), the problem of obtaining a sufficient number of well-characterized 5′ and 3′ deletions remains a serious stumbling block.

To circumvent this problem, Luckow et al. (1987) developed an alternative strategy for constructing linker-scanning mutants that does not require the matching of specific pairs of 5′ and 3′ deletion mutants. The method, which is described in detail below, involves the introduction of a nick or gap into the target DNA fragment by sequential treatment with formic acid and exonuclease III. Synthetic octanucleotide DNA linkers are then inserted at the site of the nick or gap, and a novel method is used to identify and clone target DNA fragments in which exactly 8 base pairs have been removed and replaced with the synthetic DNA linker. There are also other possible solutions to this problem. For example, the use of synthetic linkers can be eliminated by employing oligonucleotide-mediated, site-directed mutagenesis (see page 15.51) to generate a set of scanning deletion mutants, each of which lacks a precisely defined set of nucleotides. Alternatively, unidirectional deletion mutants with precisely defined 3′ and 5′ endpoints can be generated in polymerase chain reactions (see Chapter 14). If the priming oligonucleotides are designed so that they carry a restriction site at their 5′ termini, the resulting unidirectional mutations can then be converted to linker-scanning mutations essentially as described by McKnight and Kingsbury (1982). Either of these alternatives may be preferable to the protocol devised by Luckow et al. (1987).

The steps in the method of Luckow et al. (1987) are outlined in Figure 15.6. The target DNA is introduced into the polycloning site of a plasmid vector such as pUC118 or pUC119. This plasmid is then treated briefly with formic acid under conditions that allow depurination of no more than one base (on average) within the plasmid DNA. The partially depurinated DNA is then treated with exonuclease III, which recognizes and excises depurinated bases, resulting in the generation of single-strand nicks or gaps. The complementary DNA strand is then cleaved opposite the nick or gap by digestion with nuclease S1. This usually results in the removal of 0–30 nucleotides from each DNA strand at the site of the nick and generates a population of circularly permuted linear molecules that are a few nucleotides shorter than the original DNA. After the termini are repaired with bacteriophage T4 DNA polymerase, synthetic oligonucleotide linkers are ligated to both ends of the DNA fragment. The linkers of choice for this step are 8 nucleotides in length and contain a cleavage site for the restriction enzyme *Bgl*II[2]. After digestion with *Bgl*II, which creates cohesive termini at each end, the linear DNA is

[2] *Bgl*II linkers can be used only if the target fragment and the plasmid into which it is cloned both lack a *Bgl*II site. If this is not the case, it will be necessary to modify the ends of the fragment of DNA that carries the *kan*^{r} gene by addition of restriction enzyme recognition sites that are absent from both the target fragment and the plasmid.


Clone target DNA into a plasmid vector (e.g., pUC118 or pUC119)
Partially depurinate by treatment with formic acid
site of depurination
exonuclease III
Generates nick at site of depurination
nuclease S1
Converts nicked circular DNA to linear molecules
bacteriophage T4 DNA polymerase
Repairs frayed termini
insertion of BglII linker followed by digestion with BglII
insertion of 1.4-kb DNA fragment containing kanr gene

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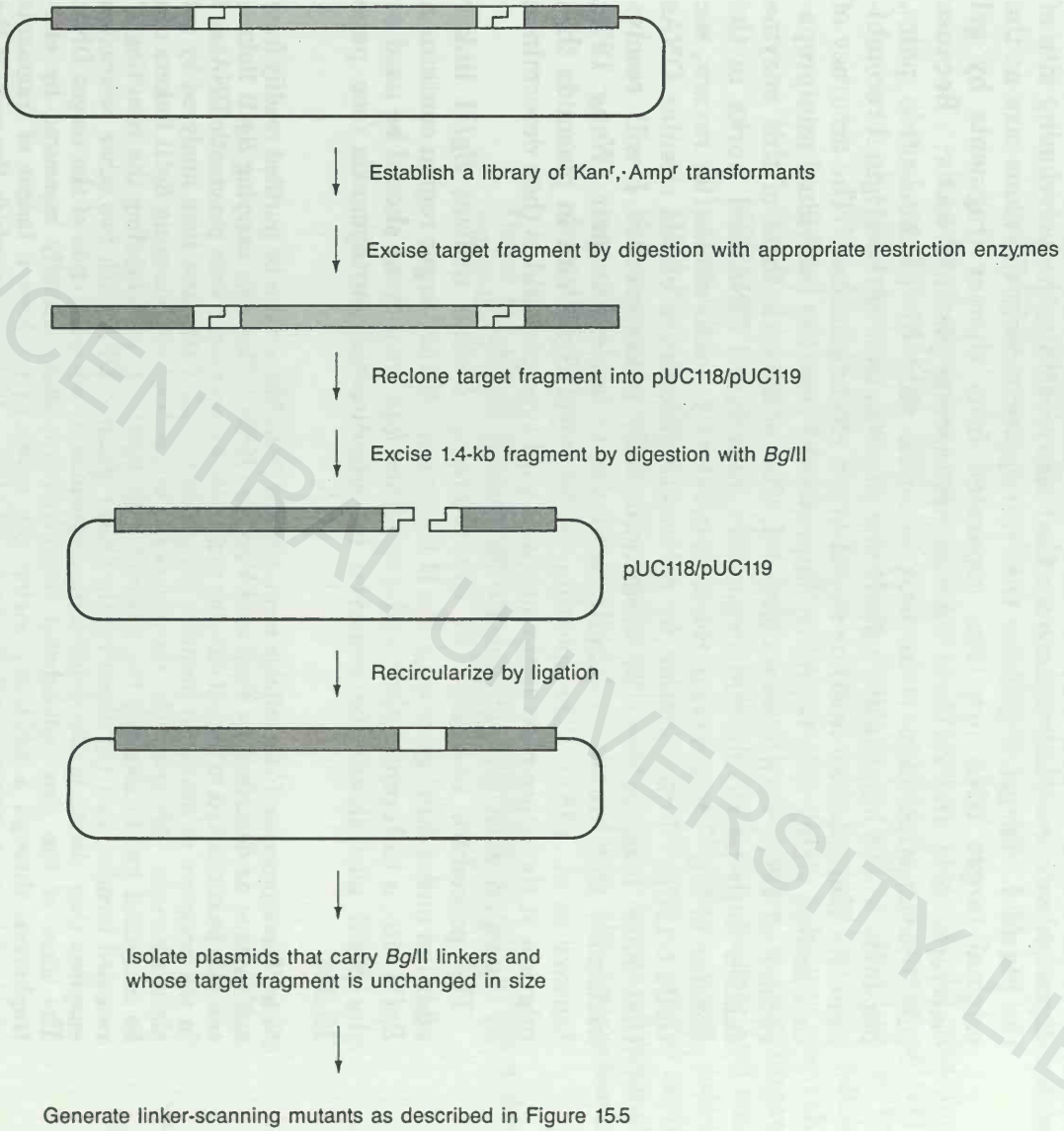
Establish a library of Kanr,·Ampr transformants
Excise target fragment by digestion with appropriate restriction enzymes
Reclone target fragment into pUC118/pUC119
Excise 1.4-kb fragment by digestion with BglII
pUC118/pUC119
Recircularize by ligation
Isolate plasmids that carry BglII linkers and whose target fragment is unchanged in size
Generate linker-scanning mutants as described in Figure 15.5

FIGURE 15.6
Insertion of linkers at sites of depurination (see text for details).

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ligated to a 1.4-kb *Bgl*II DNA fragment containing a kanamycin resistance gene (*kan*^{r}). Because only those linear DNAs containing *Bgl*II sites at each end will incorporate the *kan*^{r} gene, all bacteria transformed to kanamycin resistance by the products of ligation will carry plasmids containing a *Bgl*II site.

To recover the disrupted fragment of target DNA, plasmids isolated from the kanamycin-resistant bacteria are digested with *Bgl*II and ligated again under conditions that promote recircularization. The target DNA, which contains a single *Bgl*II linker at the site of disruption, is then recovered by digestion with restriction enzymes that cleave within the polycloning site of the plasmid. Target fragments that are approximately the same size as the original target DNA are then separated from shorter fragments by gel electrophoresis and recloned into an appropriate plasmid vector. Because this fractionation step is at best accurate only to ±5 nucleotide pairs, plasmids whose lengths are exactly the same as that of the original recombinant are identified by another round of gel electrophoresis. The accuracy of this second sizing step is greatly improved by treating individual minipreparations of DNA with topoisomerase I. The eukaryotic form of this enzyme relaxes both positively and negatively supercoiled DNA and works in the absence of Mg^{++} to generate relaxed, covalently closed circles (for review, see Gellert 1981). Fractionation by gel electrophoresis of closed circular DNAs that have been relaxed by treatment with topoisomerase I will resolve molecules that differ in length by a single nucleotide pair (Wang 1979; Luckow et al. 1987). The location of the novel *Bgl*II site in plasmids that migrate at the same rate as the original wild-type plasmid is then determined by digestion with the appropriate restriction enzymes.

The procedure described in the following section utilizes *Bgl*II linker addition and a *kan*^{r} gene on a *Bgl*II fragment. If the target region contains a *Bgl*II site, a different linker and a modified *kan*^{r} fragment should be used or the *Bgl*II site should be removed by site-directed mutagenesis (see page 15.51).

Note: Topoisomerase I is available commercially from BRL or can be purified readily from calf thymus as described by Prell and Vosberg (1980). Plasmids carrying *Bgl*II linkers can also be converted to nicked circular molecules by treatment with pancreatic DNAase I in the presence of ethidium bromide (Greenfield et al. 1975) and then analyzed by gel electrophoresis. Alternatively, the size of the target fragment carrying *Bgl*II linkers can be measured by (1) cleaving the plasmid with *Bgl*II, (2) radiolabeling the resulting 3′ recessed termini, and (3) digesting the linear plasmid DNA with two other restriction enzymes that cleave in the polycloning sequences at opposite ends of the target DNA. The sizes of the two radiolabeled fragments are then carefully measured by electrophoresis through a DNA sequencing gel, using as markers a ladder of fragments obtained by sequencing a well-characterized DNA. Plasmids in which the sum of the lengths of the radiolabeled fragments equals the length of the original target DNA are retained for further analysis by DNA sequencing (see Chapter 13).

Treatment of target DNA with formic acid

1. Dissolve 200 μg of purified superhelical plasmid DNA containing the DNA fragment to be mutagenized (target DNA) in 400 μl of distilled H_2O. Transfer the solution to a sterile, disposable 15-ml polypropylene tube. Incubate the capped tube for 5 minutes in a water bath set at 15°C.

   To monitor the progress of the depurination reaction, it is essential to use plasmid DNA that consists largely (> 90%) of superhelical molecules. This is best achieved by using a preparation that has been recently purified by equilibrium centrifugation in CsCl–ethidium bromide gradients (see Chapter 1, pages 1.42–1.44).

2. Add 40 μl of an aqueous solution of 2% formic acid (pH 2.0) that has been equilibrated to 15°C. Incubate the mixture for 4 minutes at 15°C, and then quench the depurination reaction by adding 1600 μl of 100 mM Tris · Cl (pH 7.5). Add 200 μl of 3 M sodium acetate (pH 7.0), followed by 4.5 ml of ice-cold ethanol. Mix the contents of the tube, and store the tube for 10 minutes in an ice bath. Recover the DNA by centrifugation at 5000*g* for 5 minutes at 4°C. Carefully decant the ethanol, and wash the pellet with 3 ml of 70% ethanol at room temperature. Again, recover the DNA by centrifugation, and remove the 70% ethanol by careful aspiration. Stand the open tube on the bench until the last traces of ethanol have evaporated, and then digest the depurinated DNA with exonuclease III as described on page 15.38.

   Under the conditions described in step 2, approximately 50% of the molecules of superhelical plasmid DNA should be depurinated at a single site.

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Digestion of depurinated DNA with exonuclease III

1. Dissolve the pellet of depurinated DNA (from step 2, page 15.37) in 800 μl of 1 × exonuclease III/depurination buffer. Transfer the dissolved DNA to a microfuge tube.

   As a control for the exonuclease reaction, set up two additional tubes, each containing 1 μg of the original superhelical plasmid dissolved in 20 μl of 1 × exonuclease III/depurination buffer.

1 × Exonuclease III/depurination buffer

66 mM Tris · Cl (pH 8.0)
125 mM NaCl
5 mM $CaCl_2$
10 mM dithiothreitol

$CaCl_2$ is used rather than $MgCl_2$ to suppress the exonucleolytic activity of exonuclease III. In the presence of $CaCl_2$, the enzyme efficiently cleaves the phosphodiester bond at depurinated bases but degrades sluggishly, if at all, from the resulting 3′ termini. The major products of the exonuclease reaction are therefore nicked circles and circles with a small gap a few nucleotides in length.

2. Incubate the depurinated and control DNAs for 5 minutes at 37°C. During this time, prepare three fresh microfuge tubes, each containing 30 μl of 50 mM EDTA (pH 8.0). Store these tubes in an ice bath.

3. Add 800 units of exonuclease III to the depurinated DNA and 5 units of exonuclease III to one of the control reactions. Continue incubation at 37°C. After incubating for 1 minute, transfer 270 μl of the depurinated DNA solution to one of the three microfuge tubes containing EDTA. Take further samples (270 μl) after 3 minutes and 9 minutes of incubation at 37°C.

4. After all of the samples have been transferred, immediately purify all of the DNAs by extraction with phenol:chloroform. Separate the organic and aqueous phases by centrifugation at 12,000*g* for 3 minutes at 4°C in a microfuge, and transfer the aqueous phases to fresh microfuge tubes. Precipitate the DNAs by adding 0.1 volume of 3 M sodium acetate (pH 7.0) and 2 volumes of ice-cold ethanol. Store the tubes for 10 minutes in an ice bath.

5. Collect the DNAs by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Carefully decant the ethanol, and wash the pellets of DNA with 300 μl of 70% ethanol at room temperature. Again, recover the DNAs by centrifugation, and remove the 70% ethanol by careful aspiration. Stand the open tubes on the bench until the last traces of ethanol have evaporated.

6. Dissolve each of the depurinated DNAs in 100 μl of TE (pH 7.6). Dissolve the two control DNAs in 10 μl of TE (pH 7.6). Analyze a small amount of each of the samples of DNA by electrophoresis through an agarose gel. If

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the reaction has gone well, a fraction of the depurinated DNA should have been converted to relaxed circular molecules, which migrate much slower through agarose gels than closed circular DNAs of the same size. There should be no increase in the ratio of relaxed circular molecules to superhelical molecules in the control samples.

Pool the samples of depurinated DNA that contain less than 50% relaxed circular molecules.

The exonuclease reaction is complete when all of the DNA that has been depurinated (~50% of the original closed circular plasmid DNA) has been converted to nicked circles. Conversion usually takes 1–3 minutes of incubation at 37°C.

7. Separate the mixture of nicked and gapped circles from unreacted superhelical DNA by equilibrium centrifugation in a CsCl–ethidium bromide gradient (see Chapter 1, pages 1.42–1.44). Collect the upper band of DNA, which consists of nicked circles and linear molecules, through a needle inserted through the side of the tube. Remove ethidium bromide from the DNA as described in Chapter 1, pages 1.46–1.47, recover the DNA by precipitation with ethanol, and then digest the DNA with nuclease S1 as described on pages 15.40–15.41.

It is necessary to remove superhelical plasmid DNA from the nicked circular preparation before proceeding to the next step in the protocol (digestion with nuclease S1). Superhelical DNA is under torsional strain and may therefore be susceptible to cleavage at particular sites by nuclease S1. Digestion by nuclease S1 at these sites would generate linear molecules whose termini would map at a limited number of locations on the plasmid DNA. Later in the protocol, these linear molecules would become equipped with *Bgl*II linkers and would therefore give rise to clones that carry insertions of *Bgl*II linkers only at the sites of preferential cleavage by nuclease S1.

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Digestion of the nicked/gapped circular DNA with nuclease S1

1. Digest the nicked/gapped circular DNA with nuclease S1 as follows:

   a. Redissolve the DNA purified from the CsCl–ethidium bromide gradient (60–100 μg) (step 7, page 15.39) in 500 μl of 1 × nuclease-S1 buffer (see below).

   b. Incubate the solution of DNA for 5 minutes at 37°C, add 5000 units of nuclease S1, and then continue incubation at 37°C.

   c. After incubation for 4 minutes, transfer 160 μl of the DNA solution to a microfuge tube containing 15 μl of 50 mM EDTA (pH 8.0) and 15 μl of 1 M Tris · Cl (pH 7.6).

   d. Take further samples (160 μl) after 15 minutes and 60 minutes of incubation at 37°C and place each sample in EDTA, Tris · Cl as described in c.

1 × Nuclease-S1 buffer

50 mM sodium acetate (pH 5.7)
200 mM NaCl
1 mM $ZnSO_4$
0.5% glycerol

During digestion with nuclease S1, the nicked/gapped circular plasmid DNA is linearized, and between 0 and 30 nucleotides are removed from each end of the DNA. The reaction is carried out at pH 5.7 to minimize acid-induced depurination. At this pH, nuclease S1 works rather sluggishly, and it is therefore necessary to add large amounts of the enzyme to drive the reaction to completion.

2. Purify the linear DNAs by extraction with phenol:chloroform. Separate the organic and aqueous phases by centrifugation at 12,000*g* for 3 minutes at 4°C in a microfuge, and transfer the aqueous phases to fresh microfuge tubes. Add 400 μl of ethanol to each of the three samples, and store the tubes for 10 minutes in an ice bath.

3. Collect the DNAs by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Carefully decant the ethanol, and wash the pellets of DNA with 300 μl of 70% ethanol at room temperature. Again, recover the DNAs by centrifugation, and remove the 70% ethanol by careful aspiration. Stand the open tubes on the bench until the last traces of ethanol have evaporated.

4. Dissolve each of the DNAs in 25 μl of TE (pH 7.6). Analyze a small amount of each of the samples of DNA by electrophoresis through an agarose gel, using as marker the original plasmid DNA that has been linearized by digestion with an appropriate restriction enzyme. Pool the samples that contain a significant proportion of linear DNA.

5. Repair the ends of the linear DNA with bacteriophage T4 DNA polymerase:

a. Adjust the volume of the pooled DNAs to 90 μl with TE (pH 7.6). At this stage, the pooled sample usually contains approximately 25 μg of DNA.

b. Add 10 μl of 10 × polymerase buffer (see below).

c. Add 5 units of bacteriophage T4 DNA polymerase, and incubate the reaction at room temperature for 1 minute. During this time, protruding 3′ termini are efficiently removed.

d. Add 10 μl of a solution containing all four dNTPs, each at a concentration of 2 mM. Incubate the reaction for 1 hour at 37°C.

10 × Polymerase buffer

200 mM Tris · Cl (pH 7.6)
10 mM $MgCl_2$
1 mM dithiothreitol

6. Purify the DNA by extraction with phenol:chloroform. Separate the organic and aqueous phases by centrifugation at 12,000*g* for 3 minutes at 4°C in a microfuge, and transfer the aqueous phase to a fresh microfuge tube. Add 100 μl of 5 M ammonium acetate and 400 μl of ethanol, and store the tube for 10 minutes in an ice bath.

7. Collect the DNA by centrifugation at 12,000*g* for 10 minutes at 4°C in a microfuge. Carefully decant the ethanol, and wash the pellet of DNA with 300 μl of 70% ethanol at room temperature. Again, recover the DNA by centrifugation, and remove the 70% ethanol by careful aspiration. Stand the open tube on the bench until the last traces of ethanol have evaporated.

8. Dissolve the DNA in 25 μl of TE (pH 7.6). Analyze a small amount of the DNA by electrophoresis through an agarose gel, using as marker the original plasmid DNA that has been linearized by digestion with an appropriate restriction enzyme.

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Ligation of phosphorylated synthetic Bgl*II linkers*[3]

1. Transfer 10 μg of linearized DNA ($\sim$10 pmoles of 5′ termini) (from step 8, page 15.41) and 1000 pmoles of phosphorylated *Bgl*II linkers to a fresh microfuge tube, and adjust the volume to 180 μl with TE (pH 7.6).

2. Add 20 μl of 10× ligase buffer, approximately 2 Weiss units of bacteriophage T4 DNA ligase, and 10 mM ATP to a final concentration of 1 mM. Incubate the reaction for at least 4 hours at 16°C.

10 × Ligase buffer

0.2 M Tris · Cl (pH 7.6)
50 mM $MgCl_2$
50 mM dithiothreitol

For definition of Weiss unit, see page 15.30.

3. Heat the reaction mixture to 68°C for 10 minutes to inactivate the bacteriophage T4 DNA ligase, and then add 500 units of *Bgl*II and incubate the reaction for at least 6 hours at 37°C.

4. Purify the DNA by extraction with phenol:chloroform. Separate the organic and aqueous phases by centrifugation at 12,000*g* for 3 minutes at 4°C in a microfuge, and transfer the aqueous phase to a fresh microfuge tube. Add 0.5 volume of 5 M ammonium acetate and 2.5 volumes of ethanol to the aqueous phase, and store the tube for 10 minutes in an ice bath.

5. Collect the DNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Carefully decant the ethanol, and wash the pellet of DNA with 300 μl of 70% ethanol at room temperature. Again, recover the DNA by centrifugation, and remove the 70% ethanol by careful aspiration. Stand the open tube on the bench until the last traces of ethanol have evaporated.

6. Dissolve the DNA in 20 μl of TE (pH 7.6), and analyze a small aliquot by electrophoresis through an agarose gel, using as marker the original plasmid DNA that has been linearized by digestion with an appropriate restriction enzyme. Store the DNA at −20°C until needed in step 1, page 15.44.

[3] *Bgl*II linkers can be used only if the target fragment and the plasmid into which it is cloned both lack a *Bgl*II site. If this is not the case, it will be necessary to modify the ends of the fragment of DNA that carries the *kan*^{r} gene by addition of restriction enzyme recognition sites that are absent from both the target fragment and the plasmid.

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7. Prepare a fragment of DNA carrying the *kan*^{r} gene. A convenient source of this fragment is the plasmid pBL2, which carries the *kan*^{r} gene from Tn*903* (see Luckow et al. 1987). Digest 100 μg of closed circular pBL2 DNA with *Bgl*II, and purify the 1.4-kb fragment carrying the *kan*^{r} gene by electrophoresis through a 0.8% agarose gel.

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Ligation of linearized plasmid DNA to the fragment carrying the kan^r *gene*

1. Ligate the linearized plasmid DNA (step 6, page 15.42) to the fragment carrying the *kan*^{r} gene (step 7, page 15.43). Because the efficiency with which the two fragments will ligate to each other is unknown, small pilot reactions should be set up to establish the optimum ratio of DNA fragments in the reaction. For example, dissolve 500 ng of linearized plasmid DNA and 100, 250, or 500 ng of the fragment carrying the *kan*^{r} gene (approximate molar ratios = 1:0.5, 1:1, or 1:2) in 12.5 μl of TE (pH 7.6). To each mixture, add 1.5 μl of 10 × ligase buffer (see page 15.42), 0.5 Weiss unit of bacteriophage T4 DNA ligase, and 10 mM ATP to a final concentration of 1 mM. Incubate the reactions for at least 4 hours at 16°C. Use small aliquots (2 μl of each mixture) to transform competent *E. coli* (strain DH1 or DH5) simultaneously to resistance to kanamycin and ampicillin (i.e., the bacterial plates used to select transformants should contain both kanamycin and ampicillin, each at a concentration of 100 μg/ml). Store the remainders of the ligation mixtures at −20°C until step 2 of the protocol has been completed.

   For definition of Weiss unit, see page 15.30.

   Ampicillin is included to select against plasmids in which the DNA fragment carrying the *kan*^{r} gene has been inserted into the *amp*^{r} gene rather than into the target sequence.

2. Pick 36 independent transformants, and establish small-scale (2-ml) cultures in LB medium containing ampicillin and kanamycin, each at a concentration of 100 μg/ml. After the cultures have reached saturation, isolate the plasmid DNA using one of the methods in Chapter 1. Analyze the DNAs by digestion with the appropriate restriction enzymes and gel electrophoresis to obtain an estimate of the proportion of clones that carry a *kan*^{r} gene and therefore a *Bgl*II linker in the target region.

3. From the numbers of colonies obtained and the proportion of clones with a *Bgl*II linker in the target region, determine which pilot ligation reaction(s) yielded the largest number(s) of Kan^r Amp^r colonies and calculate the amount of ligation mixture that will be required to generate 250,000 independent Kan^r Amp^r transformants that carry a *Bgl*II linker in the target region. Use this amount of ligation mixture to transform competent *E. coli* (strain DH1 or DH5). It may be necessary to repeat the ligation reaction(s) on a larger scale.

   It is best to generate these transformants in several small-scale transformation reactions rather than in a single large-scale reaction. The efficiency of transformation drops markedly when more than 200 μl of competent cells are used per reaction, probably because the timing of the heat-shock step is no longer optimal.

4. Just before plating on selective media, pool the several small-scale transformation reactions and recover the bacterial cells by centrifugation at 5000*g* for 10 minutes at room temperature. Resuspend the cells in a total volume of 2 ml of LB medium, and plate 0.5-ml aliquots of the

suspension on each of four 150-mm petri dishes containing LB agar supplemented with kanamycin and ampicillin, each at a concentration of 100 μg/ml. Incubate the plates for 18–24 hours at 37°C.

5. At the end of the incubation, add to each plate approximately 5 ml of LB medium containing ampicillin and kanamycin, each at a concentration of 100 μg/ml. Use a bent glass rod and a pasteur pipette to scrape and squirt the colonies from the surfaces of the plates. Pool the bacterial suspension obtained from each of the four plates, and recover the bacterial cells by centrifugation at 5000*g* for 10 minutes at 4°C. Remove the supernatant medium.

6. Resuspend the bacterial suspension in 5 ml of LB medium containing ampicillin and kanamycin, each at a concentration of 100 μg/ml. Use one half of the bacterial suspension to inoculate a 500-ml culture (LB medium containing ampicillin and kanamycin). Grow the culture to saturation at 37°C with agitation.

   The remainder of the bacterial suspension can be stored at −70°C as described in Appendix A.

7. Isolate and purify the pool of plasmid DNAs from the saturated 500-ml culture by one of the large-scale methods described in Chapter 1.

   There is no need to purify the pooled plasmid DNAs by equilibrium centrifugation in CsCl–ethidium bromide gradients. DNA purified by precipitation with polyethylene glycol is satisfactory (see Chapter 1, pages 1.40–1.41).

8. Recover the target DNA fragments containing the inserted *kan*^{r} gene by digesting approximately 30 μg of the pooled plasmid DNAs with the appropriate restriction enzyme (or combination of restriction enzymes).
   At this time also digest 5 μg of the original plasmid (step 1, page 15.37) with the same restriction enzyme (or combination of restriction enzymes). Purify this fragment and subclone as described in steps 9 and 10. This subclone will provide an important control in subsequent steps.

   Insertion of the fragment carrying the *kan*^{r} gene increases the size of the target fragment by approximately 1.4 kb.

9. Purify the target fragments carrying the *kan*^{r} gene by electrophoresis through a 0.7% agarose gel.

10. Ligate the purified target fragments to an appropriately prepared plasmid vector (e.g., pUC118 or pUC119). Because the efficiency with which the target fragments will ligate to the plasmid vector is unknown, small pilot reactions should be set up to establish the optimum ratio of DNA fragments in the reaction. For example, dissolve 200 ng of plasmid DNA and 0, 100, 250, or 500 ng of the target fragments in 12.5 μl of TE (pH 7.6). To each mixture, add 1.5 μl of 10× ligase buffer (see page 15.42), 0.5 Weiss unit of bacteriophage T4 DNA ligase, and 10 mM ATP to a final concentration of 1 mM. Incubate the reactions for at least 4 hours at 16°C.

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Use small aliquots (2 μl of each mixture) to transform competent *E. coli* (strain DH1 or DH5) simultaneously to resistance to kanamycin and ampicillin (i.e., the bacterial plates used to select transformants should contain both kanamycin and ampicillin, each at a concentration of 100 μg/ml). Store the remainders of the ligation mixtures at −20°C.

11. From the numbers of colonies obtained from the pilot ligations/transformations, calculate the amount of ligation mixture that will be required to generate 10,000 independent transformants that contain the target region together with its inserted *kan*^{r} gene. Use this amount to transform competent *E. coli* (strain DH1 or DH5).

It is best to generate these transformants in several small-scale transformation reactions rather than in a single large-scale reaction. The efficiency of transformation drops markedly when more than 200 μl of competent cells are used per reaction, probably because the timing of the heat-shock step is no longer optimal.

12. Just before plating on selective media, pool the several small-scale transformation reactions and recover the bacterial cells by centrifugation at 5000*g* for 10 minutes at room temperature. Resuspend the cells in a total volume of 2 ml of LB medium, and plate 0.5-ml aliquots of the suspension on each of four 150-mm petri dishes containing LB agar supplemented with kanamycin and ampicillin, each at a concentration of 100 μg/ml. Incubate the plates for 18–24 hours at 37°C.

13. At the end of the incubation, add to each plate approximately 5 ml of LB medium containing ampicillin and kanamycin, each at a concentration of 100 μg/ml. Use a bent glass rod and a pasteur pipette to scrape and squirt the colonies from the surfaces of the plates. Pool the bacterial suspension obtained from each of the four plates, and recover the bacterial cells by centrifugation at 5000*g* for 10 minutes at 4°C. Remove the supernatant medium.

14. Resuspend the bacterial suspension in 5 ml of LB medium containing ampicillin and kanamycin, each at a concentration of 100 μg/ml. Use one half of the bacterial suspension to inoculate a 500-ml culture (LB medium containing ampicillin and kanamycin). Grow the culture to saturation at 37°C with agitation.

The remainder of the bacterial suspension can be stored at −70°C as described in Appendix A.

15. Establish a 5–10-ml culture from the control plasmid subclone (step 10), and isolate the plasmid DNA by one of the methods described in Chapter 1. Verify that the subclone carries the correct fragment by restriction enzyme analysis or by DNA sequencing (see Chapter 13). The plasmid will be used as a control in step 2, page 15.47 and step 1, page 15.50.

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RECOVERY OF THE TARGET DNA FRAGMENT

1. Isolate and purify the pool of plasmid DNAs from the saturated 500-ml culture from step 14, page 15.46, by one of the large-scale methods described in Chapter 1.

   There is no need to purify the pooled plasmid DNAs by equilibrium centrifugation in CsCl–ethidium bromide gradients. DNA purified by precipitation with polyethylene glycol is satisfactory (see Chapter 1, pages 1.40–1.41).

2. To remove the fragment carrying the *kan*^{r} gene from the plasmids containing the synthetic linker, digest approximately 100 μg of purified plasmid DNAs with *Bgl*II (or the appropriate restriction enzyme if different synthetic linkers have been used). Separate the products of digestion by electrophoresis through a preparative 0.7% agarose gel, using as marker an aliquot of the control plasmid subclone (step 15, page 15.46) that has been linearized by digestion with an appropriate restriction enzyme. Excise the band of DNA that corresponds in size to the marker DNA, and extract the DNA by one of the methods described in Chapter 6.

3. Dissolve approximately 100 μg of the excised band in 100 ml of 1 × ligase buffer, and then add 200 Weiss units of bacteriophage T4 DNA ligase. Incubate the reaction for at least 4 hours at 16°C.

   1 × Ligase buffer

   20 mM Tris · Cl (pH 7.6)
   5 mM $MgCl_2$
   1 mM ATP
   5 mM dithiothreitol

   For definition of Weiss unit, see page 15.30.

4. Concentrate the DNA by extracting the ligation mixture twice with 1-butanol (see Appendix E). Add 0.2 volume of 10 M ammonium acetate, and precipitate the DNA with 2.5 volumes of ice-cold ethanol. Store the tube for 30 minutes in an ice bath.

5. Collect the DNA by high-speed centrifugation (25,000*g* for 30 minutes at 0°C). Carefully decant the ethanol, and wash the pellet of DNA with several milliliters of 70% ethanol at room temperature. Again, recover the DNA by centrifugation, and remove the 70% ethanol by careful aspiration. Stand the open tube on the bench until the last traces of ethanol have evaporated.

6. Dissolve the DNA in 200 μl of TE (pH 7.6), and estimate the recovery of DNA by analyzing a small aliquot by electrophoresis through an agarose gel.

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EXCISION OF THE TARGET DNA

1. Excise the target DNA by digesting approximately 50 μg of the recircularized plasmid DNA from step 6, page 15.47, with the appropriate restriction enzymes. After cleavage, it is necessary to fractionate the target DNA accurately to minimize the number of clones to be screened and to avoid "false-positive" clones, which have insertions or deletions of exactly 10 or 20 bp and therefore would not be eliminated in the topoisomerase screening procedure described on page 15.50.

2. Size fractionation is best achieved by two-stage electrophoresis. In the first stage, which employs a preparative 0.8% agarose gel, most of the vector DNA is removed. Load sufficient DNA on the gel to allow 4–5 μg of the target fragment to be recovered (for experimental details, see Chapter 6). Purify and concentrate the DNA recovered from the first stage, and apply it to a nondenaturing preparative (40-cm × 1-mm) gel cast with 5% polyacrylamide (for experimental details, see Chapter 6). Approximately 1 μg of target DNA can be loaded per standard 5-mm slot. As electrophoretic markers, use DNA fragments whose lengths are accurately known and whose sizes are approximately equal to that of the target DNA. Such markers can be easily generated by cleaving the original recombinant plasmid (step 1, page 15.37) with restriction enzymes that cleave at different sites in the polycloning sequences at each end of the target DNA.

3. Recover a band of DNA that differs in length from the original target fragment by no more than ±5 bp (for experimental details, see Chapter 6). Estimate the amount of DNA recovered by agarose gel electrophoresis of a small aliquot.

4. Ligate the DNA recovered from the polyacrylamide gel fragments to an appropriately prepared plasmid vector (e.g., pUC118 or pUC119). Because the efficiency with which the target DNAs will ligate to the plasmid vector is unknown, small pilot reactions should be set up to establish the optimum ratio of DNA fragments in the reaction. For example, dissolve 400 ng of plasmid DNA and 0, 10, 40, or 100 ng of the target fragments in 12.5 μl of TE (pH 7.6). To each mixture, add 1.5 μl of 10 × ligase buffer (see page 15.42), 0.5 Weiss unit of bacteriophage T4 DNA ligase, and 10 mM ATP to a final concentration of 1 mM. Incubate the reactions for at least 4 hours at 16°C. Use small aliquots (2 μl of each mixture) to transform competent *E. coli* (strain DH5α^{TM}) to resistance to ampicillin *only*. Plate the transformants on LB agar medium containing ampicillin (100 μg/ml), 4 μl of a solution of isopropylthio-β-D-galactoside (IPTG) (200 mg/ml), and 40 μl of a solution of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (20 mg/ml in dimethylformamide), and incubate them for 18–24 hours at 37°C. Store the remainders of the ligation mixtures at −20°C.

   For definition of Weiss unit, see page 15.30.

   For preparation of IPTG and X-gal solutions, see Appendix B.

5. Pick 36 independent transformants (white colonies) and establish small-scale (2-ml) cultures in LB medium containing 100 μg/ml ampicillin. After the cultures have reached saturation, isolate the plasmid DNA by one of the methods described in Chapter 1. Analyze the DNAs by digestion with the appropriate restriction enzymes and gel electrophoresis to obtain an estimate of the proportion of clones that carry a *Bgl*II linker in the target region.

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1. Set up a series of control reactions each containing 250 ng of the control superhelical plasmid (step 15, page 15.46), 1 μl of 10× topoisomerase I buffer, and TE (pH 8.0) to a final volume of 10 μl. Add no and varying amounts of topoisomerase I, and incubate the reactions for 12 hours at 37°C.

10 × Topoisomerase I buffer

100 mM Tris · Cl (pH 8.0)
2 M NaCl
0.5 M EDTA (pH 8.0)

Topoisomerase I works inefficiently on small-scale preparations of plasmid DNA, and it is therefore necessary to use far greater quantities of enzyme than normal.

Topoisomerase I is available commercially from BRL or can be purified readily from calf thymus as described by Prell and Vosberg (1980). The supercoiled DNA can also be relaxed by other methods (see note, page 15.36) before analysis by gel electrophoresis.

2. Add 12 μl of TE (pH 7.6) to each sample (to dilute the NaCl), followed by 4 μl of gel-loading buffer. Load the samples into the slots of a long (25-cm), horizontal 1.4% agarose gel cast in 1 × TAE (see Appendix B) *in the absence of ethidium bromide*. Carry out electrophoresis at 100 V (4 V/cm) for approximately 24 hours (or until the xylene cyanol FF tracking dye has migrated 20 cm). The electrophoresis buffer (1 × TAE) should not contain ethidium bromide.

To prevent changes in pH, the electrophoresis buffer should be recirculated or changed at least once during the 24-hour run.

3. When electrophoresis is complete, stain the gel for 1 hour in 1 × TAE containing ethidium bromide (0.5 μg/ml) (see Chapter 6, pages 6.15–6.17).

4. Determine the amount of topoisomerase I that is required for complete conversion of the control plasmid superhelical DNA to a completely relaxed form with a new mobility. Repeat steps 1–3 using this optimal amount of topoisomerase I and 250 ng of each of the small-scale preparations of plasmid DNA from step 5, page 15.49. Also include the control plasmid treated with topoisomerase I.

5. Store cultures containing plasmids that carry a *Bgl*II linker in the target fragment and comigrate with the original recombinant plasmid after treatment with topoisomerase I at −70°C (see Chapter 1) until needed for the next stage of analysis (usually sequencing of double-stranded DNA; see Chapter 13). Process further batches of plasmids until sufficient linker-insertion mutants are accumulated to complete the task at hand.

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Oligonucleotide-mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is used to add, delete, or substitute nucleotides in a segment of DNA whose sequence is known. In contrast to most other methods of mutagenesis, which typically spawn mixed populations of variants, oligonucleotide-mediated mutagenesis specifically generates mutations designed by the experimenter. Because of this precision, the method can be used, for example, to alter individual codons in protein coding sequences or to generate defined changes in sequences that have a regulatory function. In addition, oligonucleotide-mediated mutagenesis can be used to facilitate construction of new vectors and chimeric genes. For example, sequences such as ribosome-binding sites or polyadenylation signals can be inserted at predetermined positions in expression vectors; inconvenient restriction sites can be removed and convenient sites can be added at specified positions; and, finally, undesirable sequences (e.g., introns and DNA sequences that code for untranslated regions of mRNA) can be eliminated and different domains (e.g., promoters and coding regions) can be linked together with precision.

The feasibility of introducing specific changes at defined locations in DNA was first recognized in the early 1970s from work aimed at mapping the locations of mutations on the single-stranded genome of the small bacteriophage ϕX174. When fragments of denatured wild-type bacteriophage DNA were transfected into susceptible bacteria together with intact single-stranded bacteriophage DNA carrying an amber mutation, "marker rescue" was observed, i.e., bacteriophages carrying wild-type genomes were generated. Marker rescue occurred in the transfected bacteria because the fragment of wild-type DNA annealed to the corresponding sequence of the amber mutant, forming a mismatched heteroduplex that was converted by host-specified mismatch-repair systems into a full-length, wild-type genome. It was quickly realized that this process could also be used in reverse, i.e., that specific mutations could be introduced into wild-type DNA using mutated double-stranded fragments of viral DNA (Weisbeek and Van de Pol 1970; Hutchison and Edgell 1971). Later, when pioneering work in DNA chemistry had led to the routine synthesis of oligonucleotides (Letsinger and Lunsford 1976; Khorana 1979; Matteucci and Caruthers 1981), and when the availability and quality of DNA-modifying enzymes (DNA polymerase and DNA ligase) had improved, in vitro techniques for oligonucleotide-mediated DNA mutagenesis were developed. The first methods used synthetic oligonucleotides that were completely homologous to single-stranded bacteriophage ϕX174 DNA except for a single base change that, if incorporated into the bacteriophage genome, would generate a selectable phenotype. The oligonucleotides were annealed to single-stranded bacteriophage ϕX174 DNA and used as primers for DNA synthesis catalyzed in vitro by the Klenow fragment of *E. coli* DNA polymerase I. When the resulting heteroduplexes were transfected into bacteria, a dramatic increase was observed in the frequency of bacteriophages displaying the desired phenotype (Hutchison et al. 1978; Razin et al. 1978).

Many of the methods in current use for oligonucleotide-mediated mutagenesis differ little in principle from the techniques used originally by Hutchison and his colleagues. However, there has been a succession of significant improvements to virtually all technical aspects of the procedure. These include: (1) a better understanding of the conditions for hybridization of mismatched oligonucleotides (Wallace et al. 1979), (2) a significant increase in the efficiency with which mismatched heteroduplexes can be generated in vitro (for review, see Smith 1985), (3) the development of techniques to screen bacteriophage plaques for the desired mutation by oligonucleotide hybridization (Wallace et al. 1979, 1981b), (4) the development of a versatile set of bacteriophage M13 vectors (for review, see Chapter 4), and (5) the invention of genetic methods to reduce the frequency of bacteriophages that carry nonmutant DNAs (see, e.g., Kunkel 1985). As a consequence of these improvements, which are reviewed in the following pages, it is now possible to introduce virtually any desired change into a known sequence of DNA. In many cases, oligonucleotide-mediated mutagenesis is the only method by which such changes can be created.

Protocols for oligonucleotide-mediated mutagenesis usually involve the following steps:

1. Cloning of an appropriate fragment of DNA into a bacteriophage M13 vector
2. Preparation of single-stranded DNA from the recombinant bacteriophage M13
3. Design and synthesis of mutagenic oligonucleotides
4. Hybridization of mutagenic oligonucleotides to the target DNA
5. Extension of the hybridized oligonucleotide by DNA polymerase
6. Transfection of susceptible bacteria
7. Screening of bacteriophage plaques for those carrying the desired mutation
8. Preparation of single-stranded DNA from the mutagenized recombinant bacteriophage
9. Confirmation by sequencing that the mutagenized bacteriophage M13 DNA carries the desired mutation and no other
10. Recovery of the mutated fragment of DNA from the double-stranded replicative form of the recombinant bacteriophage M13
11. Substitution of the mutagenized fragment for the corresponding segment of wild-type DNA

Many of these steps are discussed in detail in other chapters of this manual. Chapter 4, for example, contains methods that are used to clone fragments of DNA into bacteriophage M13 vectors, to prepare single-stranded templates, etc. In the remainder of this section, therefore, we discuss only those techniques that are specific to site-directed mutagenesis.

Preparation of Single-stranded Target DNA

All oligonucleotide-mediated mutagenesis procedures require a target DNA that is at least partially single-stranded. This can be prepared simply and efficiently by cloning the target DNA into bacteriophage M13 or into recombinant plasmids (phagemids) containing origins of replication derived from single-stranded bacteriophages (see Chapter 4). There are good reasons why the segment of target DNA cloned into these vectors should be as small as possible:

- Large segments of DNA can be unstable in single-stranded bacteriophage vectors and are prone to suffer spontaneous deletion.
- The chance that the mutagenic oligonucleotide will hybridize to an inappropriate site rather than to a desired sequence increases as the size of the target DNA increases.
- To ensure that the mutagenized target DNA contains only the desired mutation and no other, it is essential to sequence the entire fragment after oligonucleotide-mediated mutagenesis has been completed. The shorter the target DNA, the easier the task of determining its entire sequence.

In most instances, naturally occurring restriction sites can be used to insert an appropriately sized segment of target DNA (< 1 kb) into a single-stranded DNA vector. In a few cases, however, when no suitable restriction sites are present, it may be necessary to consider other options. These include:

- Cloning a fragment of target DNA that is larger than optimal.
- Carrying out preliminary oligonucleotide-mediated mutagenesis to introduce restriction site(s) at appropriate locations in the target DNA. This is worthwhile when many oligonucleotide-mediated mutations are to be introduced into the same region of DNA, for example, when generating a comprehensive set of substitutions of a particular amino acid. Under such circumstances, it is usually possible to take advantage of the degeneracy of the genetic code to introduce novel restriction site(s) upstream of or downstream from the target area without altering the sequence of amino acids for which the DNA codes. The resulting "cassette" of DNA can then be easily shuttled in and out of the bacteriophage M13 vector used for oligonucleotide-mediated mutagenesis.
- Avoiding bacteriophage M13 vectors altogether. Mutagenesis is then carried out using double-stranded DNA derived from a plasmid. A number of methods have been developed to avoid the use of single-stranded vectors, but all of them are comparatively inefficient and should only be used in desperation. These methods include: (1) exonucleolytic digestion of plasmid DNA that has been nicked at a specific site (Wallace et al. 1981a; Efimov et al. 1985), (2) denaturation of supercoiled DNA (Schold et al. 1984), and (3) formation of heteroduplexes between two linear DNA fragments such that the resulting molecule is circular and has a single-strand gap that includes the target site for mutagenesis (Oostra et al. 1983; Morinaga et al. 1984).

Design and Selection of Mutagenic Oligonucleotides

Oligonucleotides used for site-directed mutagenesis must have the following two properties: (1) They must be complementary to the appropriate strand of the target DNA, and (2) they must be sufficiently long to anneal specifically to the target sequences. In addition, mutagenic oligonucleotides should be designed, whenever possible, to be free of palindromic, reiterated, or self-complementary sequences that might form stable secondary structures. The greater the potential for such structures, the lower the efficiency with which the oligonucleotide will hybridize to its target sequence in the template DNA. In extremely rare cases, the secondary structure of the oligonucleotide (or its complementary target sequence) is so great that no mutants can be obtained. Under such circumstances, it may be necessary to carry out mutagenesis with a much longer synthetic oligonucleotide whose 5′ and 3′ termini extend well beyond (15–20 nucleotides) the problem region. Screening for the desired mutants is then carried out with a much smaller oligonucleotide (17–19 nucleotides) that carries the potentially mismatched base(s) in the central position.

The sequence of mutagenic oligonucleotides should also be analyzed for the ability to form fortuitous, stable hybrids with (1) the (+) and (−) strands of the bacteriophage M13 vector DNA and (2) regions of the target DNA other than the sequences used to design the oligonucleotides. Most commonly available programs for computer analysis of DNA sequences can be used for this purpose. If there is significant homology—a run of eight or more perfectly matched bases between the mutagenic oligonucleotide and sequences in the bacteriophage M13 vector or the target DNA—the specificity of hybridization of the mutagenic oligonucleotide can be checked either in a dideoxy-mediated sequencing reaction in which the oligonucleotide is used as a primer or by extending the primer with labeled nucleotides and then digesting the product with appropriate restriction enzymes. The pattern of radioactive bands will reveal whether priming has occurred at the desired site and the length to which the primer has been extended (Zoller and Smith 1984). However, tests of this type are generally carried out only when difficulties are encountered in obtaining the desired mutation.

The design of mutagenic oligonucleotides is dictated by the type of mutation that is to be constructed.

1. *Oligonucleotides used for substitution, addition, or deletion of single nucleotides.* Typically, these oligonucleotides are 17–19 nucleotides in length and carry the mismatched nucleotide at the center or at one of the two nucleotide positions immediately 3′ of the center. This design is optimal for several reasons:

   - The 5′ terminus of the oligonucleotide forms a perfect hybrid with the template, so that DNA synthesis initiated from an upstream primer (see below) does not easily displace the mutagenic oligonucleotide. Approximately 8–10 perfectly matched nucleotides are required to suppress displacement of the oligonucleotide by the Klenow fragment of *E. coli* DNA polymerase I. Displacement can also be prevented by using enzymes such as bacteriophage T4 DNA polymerase (Nossal 1974; Kunkel

1985) or Sequenase™ (Schena 1989), which cannot readily remove the hybridized mutagenic primer from its template.

- The hybrid formed between the template and the 3′ terminus of the oligonucleotide is sufficiently stable to allow priming of DNA synthesis to occur with high efficiency. If the mismatched nucleotide is too close to the 3′ terminus, the 3′ segment of the oligonucleotide will be unable to form a stable hybrid with the target DNA and will therefore be susceptible to exonucleolytic degradation if the Klenow fragment of *E. coli* DNA polymerase I is used in the primer-extension reaction (Gillam and Smith 1979). In addition, an increase in the frequency of priming at incorrect locations might occur because the unhybridized 3′ region of the mutagenic oligonucleotide is now free to anneal to incorrect sites on the template. To suppress these effects, between 7 and 9 perfectly matched nucleotides are required at the 3′ terminus of the mutagenic oligonucleotide.
- The difference in thermal stability between perfectly matched hybrids and mismatched hybrids is sufficiently great that the mutagenic oligonucleotide can be used to screen bacteriophage M13 plaques by hybridization for potential mutants. As discussed in Chapter 11, the longer the oligonucleotide, the smaller the difference in thermal stability between a perfectly matched hybrid and one containing a single mismatched base pair. The aim, therefore, is to use the shortest mutagenic oligonucleotide that, under the conditions used for primer extension, will form stable hybrids both upstream of and downstream from the mismatch. Under normal circumstances, the mutagenic oligonucleotide should be between 17 and 19 nucleotides in length, with the mismatch centrally located.

2. *Oligonucleotides used to create deletions or insertions or to substitute two or more contiguous nucleotides.* Oligonucleotides 25 or more nucleotides in length are used to insert, delete, or substitute two or more bases. Optimally, there should be 12–15 perfectly matched nucleotides on either side of the central looped-out region to ensure that both ends of the mutagenic oligonucleotide are stably hybridized at the temperature used for primer extension. The thermal stability of each of the two flanking regions can be estimated from the following formula:

$$T_m = 4(G + C) + 2(A + T)$$

where T_m = melting temperature (in 6 × SSC), G = number of G residues in the sequence, C = number of C residues in the sequence, A = number of A residues in the sequence, and T = number of T residues in the sequence. Mutagenesis occurs efficiently when the T_m of each of the double-stranded regions flanking the mismatched or looped-out sequence is approximately 35–40°C.

In general, the larger the size of the mutation to be constructed, the lower the efficiency of oligonucleotide-mediated mutagenesis. This inefficiency stems from two sources. First, the ability of the mutagenic oligonucleotide to form stable hybrids with two separate sequences on the

template DNA decreases as a function of the distance between the two sequences. Second, there is an increased probability that the oligonucleotide will hybridize to incorrect sequences on the template DNA. The consequence of these effects is that the efficiency of "loop-in/loop-out" mutagenesis is far lower than the efficiency of mutagenesis of one or two contiguous nucleotides. Furthermore, the proportion of bacteriophages that carry alterations at ectopic sites is greatly increased. It is therefore *essential* to determine the complete sequence of the mutagenized DNA (not just the region that is the planned target).

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Hybridization of Oligonucleotides to the Template DNA and Primer Extension

In the standard double-primer method (Norris et al. 1983; Zoller and Smith 1984, 1987) (see Figure 15.7), two oligonucleotides—the phosphorylated mutagenic oligonucleotide and a universal sequencing primer (which need not be phosphorylated)—are mixed in a 10- to 50-fold molar excess with the single-stranded template DNA in a small volume of buffer. The template and oligonucleotides are heated briefly to 20°C above the estimated T_m, to denature any regions of secondary structure, and then cooled slowly to room temperature. Hybrids form as the temperature of the reaction mixture drops below the relevant T_m. The stoichiometry of the reagents in the mixture ensures that virtually all of the single-stranded template DNA is driven into hybrids with both oligonucleotides.

This protocol works well for mutagenic oligonucleotides of a wide variety of lengths and base compositions. However, annealing mixtures containing mutagenic oligonucleotides that are exceptionally rich in A + T may need to be cooled to lower temperatures (12–16°C) in order to stabilize the hybrids.

After the annealing reaction is complete, a mixture containing a DNA polymerase, dNTPs, DNA ligase, and ATP is added, and primer extension is allowed to proceed for 2–15 hours at the appropriate temperature for the polymerase being used. DNA synthesis is initiated both at the 3′ terminus of the mutagenic oligonucleotide and at the 3′ terminus of the upstream universal sequencing primer. The sequencing primer is extended by DNA polymerase until the 3′ terminus of the newly synthesized strand encounters the 5′ terminus of the phosphorylated mutagenic oligonucleotide (see Figure 15.7). The two segments of DNA then become ligated by the action of bacteriophage T4 DNA ligase. This sealing of the phosphodiester bond protects the 5′ terminus of the mutagenic oligonucleotide from displacement by the newly growing strand and prevents 5′ exonucleolytic editing of mismatched nucleotides after the DNA is transfected into *E. coli*. The 3′ terminus of the mutagenic oligonucleotide is also extended, resulting in the formation of a mutant wild-type heteroduplex (see Figure 15.7) that is transfected into the *E. coli*.

Any of several different DNA polymerases may be used in the extension reaction. Until recently, most workers relied exclusively on the Klenow fragment of *E. coli* DNA polymerase I, which lacks 5′ exonucleolytic activity and is therefore incapable of degrading the mutagenic oligonucleotide. Although the efficiency of mutagenesis is reported to be higher when bacteriophage T4 DNA polymerase (Geisselsoder et al. 1987) or Sequenase (Schena 1989) is used, the Klenow fragment generally gives yields of mutants that are more than adequate, and this remains the enzyme of choice.

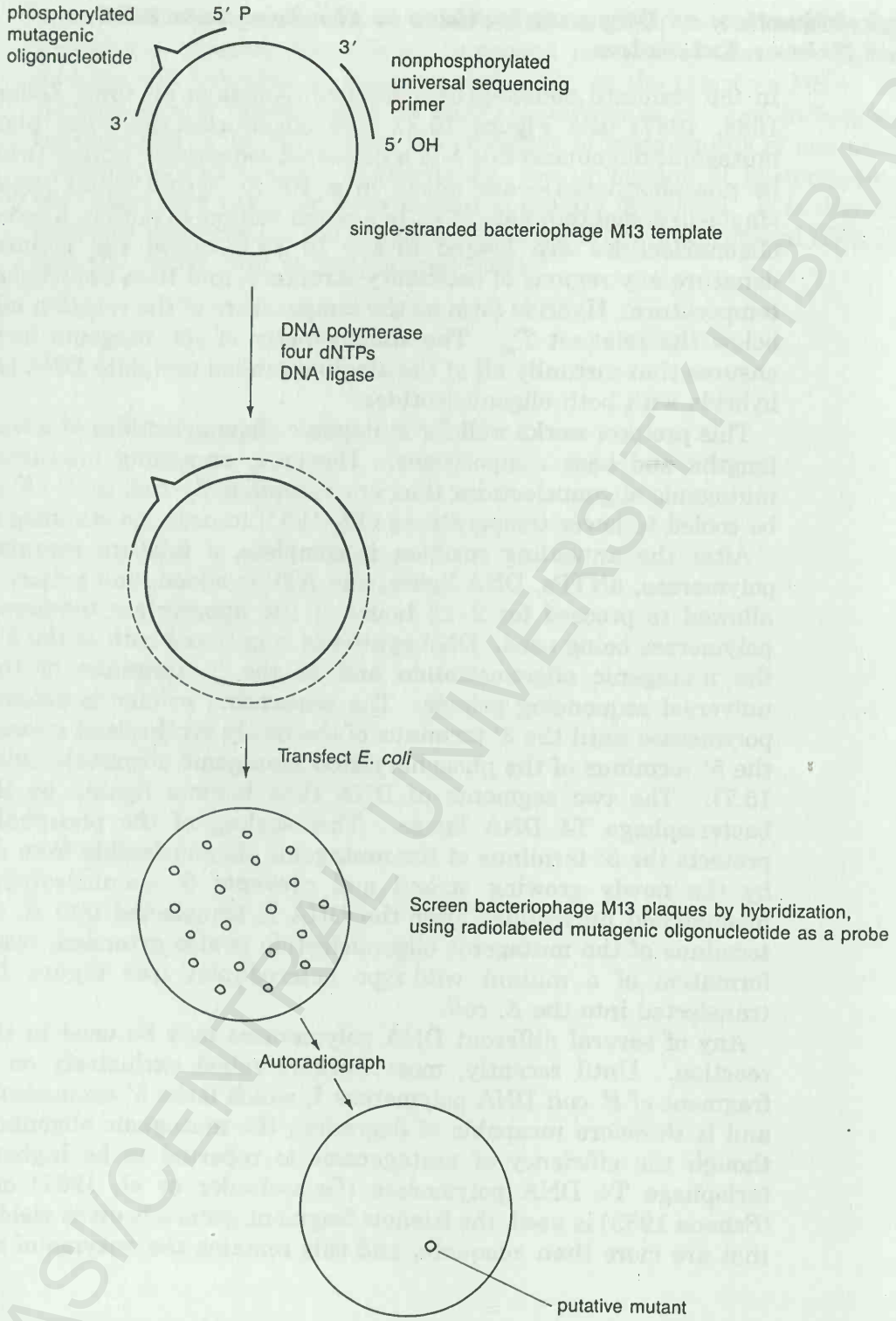

FIGURE 15.7
Oligonucleotide-mediated, site-directed mutagenesis using the double-primer method (see text for details).

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Transfection of E. coli and Screening for Mutants

After the primer-extension reaction is complete, the resulting mixture of double-stranded heteroduplex DNA is then transfected directly into an appropriate bacterial host. Most of the transfected cells release bacteriophage particles that carry a wild-type copy of the target fragment. However, a portion of the transfected cells generate particles whose genomes carry the desired mutation. Mutants can therefore be identified by screening plaques by hybridization, using the radiolabeled mutagenic oligonucleotide as a probe.

Earlier protocols (see, e.g., Zoller and Smith 1982, 1983), in which the extension reaction was primed by a single, phosphorylated, mutagenic oligonucleotide, called for the purification of covalently closed circular DNA before transfection. The aim of this step was to reduce the background of plaques containing wild-type target DNA. However, this time-consuming enrichment procedure is no longer necessary because of the improvement in efficiency of mutagenesis brought about by the use of double primers (see page 15.57). In addition, advances in the understanding of the hybridization properties of small oligonucleotides (reviewed in Chapter 11) have made it possible to screen rapidly and simultaneously many thousands of plaques to identify those generated by bacteriophages carrying the desired mutation. Hybridization is usually carried out under conditions that allow the radiolabeled oligonucleotide to form hybrids with both mutant and wild-type DNA. By progressively increasing the stringency of the subsequent washes, it is almost always possible to find conditions that (1) cause dissociation of mismatched hybrids, such as those formed between the mutagenic oligonucleotide and wild-type DNA, and (2) do not dissociate perfect hybrids formed by the oligonucleotide and the desired mutant.

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Recovery of the Mutated Fragment of DNA

Putative mutants that have been identified by hybridization are plaque-purified and rescreened by hybridization. Single-stranded DNA is then prepared from individual candidate plaques, and the segment of foreign DNA is sequenced in two stages. First, the presence of the desired mutation is confirmed—usually by the dideoxy-mediated chain-termination method. In those cases where the target site for mutation lies a considerable distance downstream from the binding site for universal sequencing primer, it may be necessary to use a synthetic primer complementary to a sequence that lies closer to the mutation site. Second, once the presence of the desired mutation has been confirmed, the entire segment of foreign DNA carried in the vector is sequenced to eliminate the possibility of adventitious mutations at ectopic sites.

Restriction enzymes are used to recover the foreign DNA (or a subfragment carrying the desired mutation) from the double-stranded replicative form of the recombinant bacteriophage genome that has been sequenced. The mutated fragment is then used to replace the homologous segment of the wild-type DNA, and the phenotypic effects of the mutation are measured by the appropriate assays.

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Methods to Improve the Efficiency of Oligonucleotide-mediated Mutagenesis

The basic procedures described above have been used successfully for several years to isolate a wide variety of site-directed mutants. Occasionally, however, difficulties are encountered in obtaining a particular mutation by the standard procedure. These difficulties have several causes:

- *The nature of the mutation itself.* The larger and more complex the mutation, the lower the efficiency with which it will be generated. For example, large deletions (i.e., deletions of several hundred nucleotides) are generated with approximately 50-fold lower efficiency than mutations involving only local changes in sequence. This decrease almost certainly occurs because the mutagenic oligonucleotide has problems in forming and maintaining stable hybrids with widely separated tracts of target DNA.
- *The nature of the target sequences.* Regions of single-stranded target DNA with a high propensity to form stable secondary structures (hairpin loops, stem loops, etc.) are difficult to mutagenize, presumably because such structures reduce the efficiency of annealing of the mutagenic oligonucleotide. A related problem may arise when the target DNA consists of, or contains, repeated sequences. In these cases, the mutagenic oligonucleotide may be able to anneal to sequences present at more than one location in the target DNA and may generate additional mutations at these sites. Thus, only a fraction of the plaques that hybridize to the oligonucleotide probe may actually carry the desired mutation.
- *The nature of the vector.* Oligonucleotide-mediated mutagenesis is generally carried out with templates generated from recombinant M13 bacteriophages. However, single-stranded templates can also be obtained by superinfection of bacteria that have been transformed with plasmids (phagemids) carrying an origin of DNA replication derived from a single-stranded bacteriophage vector (see Chapter 4). Although the phagemid system bypasses two time-consuming steps—cloning the fragment of target DNA into a bacteriophage M13 vector and recovering it after mutagenesis—the overall efficiency of mutagenesis is reduced by approximately five- to tenfold. This inefficiency results largely from variation in yield of single-stranded DNA from one superinfection experiment to the next and from one phagemid clone to another. Although the phagemid system can generally be used to isolate simple mutants (containing point mutations and small deletions or insertions), bacteriophage M13 vectors are preferred when constructing more complicated mutants.

To improve the efficiency with which such recalcitrant mutants can be isolated, a large number of variations of site-directed mutagenesis have been described (see, e.g., Marmenout et al. 1984; Bauer et al. 1985; Carter et al. 1985; Smith 1985; Taylor et al. 1985; Carter 1987; Zoller and Smith 1987). The best of these methods, however, was developed by Kunkel (1985; Kunkel et al. 1987) (see page 15.74) and takes advantage of a strong biological selection that can be applied against the wild-type strand of DNA used as template in oligonucleotide-mediated, site-directed mutagenesis. Because of

this selection, most of the bacteriophage plaques obtained are generated by strands of DNA that are synthesized in vitro and primed by the mutagenic oligonucleotide. Although the *total number* of plaques obtained in the Kunkel method is much lower than the number obtained by more traditional mutagenesis procedures, the *proportion* of plaques that are generated by bacteriophages carrying the desired mutation is much greater. Kunkel's method is therefore valuable under two circumstances: (1) when attempting to isolate "difficult" mutants that are generated with low efficiency in the in vitro primer-extension reaction and (2) when using pools of mutagenic oligonucleotides to generate many mutants simultaneously at a defined location in the target DNA. Because a large proportion of the bacteriophages generated by the Kunkel method carry a mutation, there is generally no need to screen plaques by hybridization. Instead, single-stranded bacteriophage DNAs generated by individual plaques can be analyzed directly by DNA sequencing. This is an obvious advantage when attempting to isolate canonical sets of mutants at particular sites in the target DNA.

Protocols for the standard method of oligonucleotide-mediated mutagenesis and for the Kunkel method are presented below.

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OLIGONUCLEOTIDE-MEDIATED MUTAGENESIS BY THE DOUBLE-PRIMER METHOD

The following method is modified from Zoller and Smith (1987).

1. In preparation for mutagenesis, clone a small fragment of DNA carrying the target sequence into an appropriate bacteriophage M13 vector (usually bacteriophage M13mp18 or M13mp19). Prepare single-stranded template DNA from a plaque generated by the recombinant bacteriophage. Methods for cloning into bacteriophage M13 vectors and for preparation of single-stranded bacteriophage DNA are given in Chapter 4.

   The mutagenic oligonucleotide should be designed as described on pages 15.54–15.56 and should be complementary to the strand of the target DNA that is packaged in bacteriophage M13 particles [the (+) strand]. Before use in site-directed mutagenesis, the mutagenic oligonucleotide should be purified by Sep-Pak C_{18} column chromatography to remove salts and other impurities (see Chapter 11, page 11.39). However, it is generally not necessary to purify the oligonucleotide by polyacrylamide gel electrophoresis unless the oligonucleotide is more than 30 nucleotides in length or is to be used for "loop-in" or "loop-out" mutagenesis.

2. Phosphorylate the mutagenic oligonucleotide with bacteriophage T4 polynucleotide kinase. Mix:

mutagenic oligonucleotide	100–200 pmoles
H_2O to 16.5 μl	
10 × bacteriophage T4 polynucleotide kinase buffer	2 μl
10 mM ATP	1 μl
bacteriophage T4 polynucleotide kinase	4 units

   Incubate the reaction for 1 hour at 37°C, and then heat the reaction for 10 minutes at 68°C to inactivate the polynucleotide kinase.

10 × Bacteriophage T4 polynucleotide kinase buffer

0.5 M Tris · Cl (pH 7.6)
0.1 M $MgCl_2$
50 mM dithiothreitol
1 mM spermidine HCl
1 mM EDTA (pH 8.0)

3. Anneal the phosphorylated mutagenic oligonucleotide and nonphosphorylated universal sequencing primer to the single-stranded bacteriophage M13 DNA containing the target sequence. Mix:

single-stranded template DNA (~1 μg)	0.5 pmole
phosphorylated mutagenic oligonucleotide	10 pmoles
nonphosphorylated universal sequencing primer	10 pmoles
10 × PE1 buffer	1 μl
H_2O to 10 μl	

The molar ratio of primer to template should be between 10:1 and 50:1. Using more primer will increase the frequency of mutation at ectopic sites in the target DNA.

10 × PE1 buffer

200 mM Tris · Cl (pH 7.5)
100 mM $MgCl_2$
500 mM NaCl
10 mM dithiothreitol

Heat the mixture for 5 minutes to 20°C above the theoretical T_m of a perfect hybrid formed by the mutagenic oligonucleotide, calculated from the formula $T_m = 4(G + C) + 2(A + T)$.

Transfer the tube containing the reaction mixture to a beaker containing water at $T_m + 20°C$. Stand the beaker on the bench, and allow the reaction to cool slowly to room temperature (~20 minutes). Centrifuge the tube briefly (5 seconds) in a microfuge to collect any fluid that has condensed on the walls of the tube.

4. Prepare the following reactants (PE3 mixture) while the reaction cools to room temperature. Mix:

10 × PE2 buffer	1.0 μl
a solution containing all four dNTPs, each at a concentration of 2 mM	1.0 μl
10 mM ATP	1.0 μl
H_2O to 8.5 μl	
bacteriophage T4 DNA ligase	5 Weiss units
Klenow fragment of *E. coli* DNA polymerase I	2.5 units

Store the PE3 mixture on ice until it is needed.

10 × PE2 buffer

200 mM Tris · Cl (pH 7.5)
100 mM $MgCl_2$
100 mM dithiothreitol

For definition of Weiss unit, see page 15.30.

Any of several different DNA polymerases may be used in the extension reaction. Until recently, most workers relied exclusively on the Klenow fragment of *E. coli* DNA polymerase I, which lacks 5′ exonucleolytic activity and is therefore incapable of degrading the mutagenic oligonucleotide. Although the efficiency of mutagenesis is reportedly higher when bacteriophage T4 DNA polymerase (Geisselsoder et al. 1987) or Sequenase (Schena 1989) is used, the Klenow fragment generally gives yields of mutants that are more than adequate. *However*, if a single phosphorylated mutagenic oligonucleotide is used as a primer (e.g., in the Kunkel method of site-directed mutagenesis), bacteriophage T4 DNA polymerase (Nossal 1974; Kunkel 1985) or Sequenase (Schena 1989) *should be used* in the polymerization/extension reaction. Unlike the Klenow fragment of *E. coli* DNA polymerase I, neither of these enzymes is

able to displace the mutagenic oligonucleotide from its template. Use dNTPs of the highest quality to minimize the possibility that contaminating dUTP might be incorporated into the newly synthesized strand of DNA. The concentrated dNTP solutions sold by Pharmacia have worked well in our hands.

When using bacteriophage T4 DNA polymerase or Sequenase, the extension reaction should be incubated for 5 minutes at 0°C, 5 minutes at room temperature, and then 2 hours at 37°C. The low temperature optimizes initiation of DNA synthesis from the 3′ terminus, and the subsequent incubation at 37°C improves the efficiency of the extension reaction. In addition, the concentration of each of the four dNTPs in the reaction should be increased to 500 μM when using these polymerases. This increases the efficiency of the extension reaction and suppresses the strong 3′ exonuclease activity of bacteriophage T4 DNA polymerase.

5. Add 10 μl of the ice-cold PE3 mixture to the reaction mixture containing single-stranded DNA and annealed oligonucleotides (step 3). Incubate the final reaction mixture for 6–15 hours at 16°C.

6. Transfect competent *E. coli* of an appropriate host strain (e.g., TG1) as follows:

   a. Make a series of dilutions of the reaction mixture (1:10, 1:100, and 1:500) in 10 mM Tris · Cl (pH 7.6).

   b. In Falcon 2059 tubes, precooled to 0°C, combine 1- and 5-μl aliquots of the undiluted reaction mixture and of each dilution of the reaction mixture with 200-μl aliquots of competent TG1 cells (prepared as described in Chapter 1, pages 1.76–1.81).

   c. Store the mixtures on ice for 30 minutes, and then transfer them for exactly 2 minutes to a water bath equilibrated at 42°C.

   d. Remove the transfected cultures from the water bath, and mix each of them with 100 μl of an overnight culture of TG1 cells.

      There is no need to add TG1 cells if freshly prepared, rather than frozen, competent cultures are used.

   e. Add 2.5 ml of 2× YT top agar (melted and cooled to 45°C) to each culture, and plate each mixture on a separate YT agar plate. Incubate the plates for 16 hours at 37°C to allow plaques to form.

   If mutagenesis is carried out by the Kunkel method (see pages 15.74–15.79), 1-μl and 5-μl aliquots of the undiluted reaction mixture are used to transfect competent cultures of *E. coli* strain TG1.

   If single-stranded DNA derived from a phagemid such as pUC118 or pUC119 is used as the template for mutagenesis, 1-μl and 5-μl aliquots of the undiluted reaction mixture are used to transform competent cultures of *E. coli* strain MV1184 (see Chapter 4, page 4.15, for a description of this strain and Chapter 4, pages 4.37–4.38, for a description of the transformation protocol). Plate 50-μl and 100-μl aliquots of each transformation mixture onto LB agar plates containing 50 μg/ml ampicillin. Ampicillin-resistant colonies should appear after 18–24 hours of incubation at 37°C. Screen the transformed colonies as described on pages 15.72–15.73.

7. Screen plaques (pages 15.68–15.71) or colonies (pages 15.72–15.73) by hybridization with a radiolabeled oligonucleotide probe to detect putative mutants.

SCREENING BACTERIOPHAGE M13 PLAQUES AND BACTERIAL COLONIES BY HYBRIDIZATION TO RADIOLABELED OLIGONUCLEOTIDES

Radiolabeling of Oligonucleotides by Phosphorylation

1. Set up a reaction mixture as follows:

mutagenic oligonucleotide (10 pmoles/μl)	1.0 μl
10 × bacteriophage T4 polynucleotide kinase buffer	2.0 μl
[γ-^{32}P]ATP (sp. act. 5000 Ci/mmole; 10 mCi/ml in aqueous solution)	5.0 μl
H_2O	11.4 μl

10 × Bacteriophage T4 polynucleotide kinase buffer

0.5 M Tris · Cl (pH 7.6)
0.1 M $MgCl_2$
50 mM dithiothreitol
1 mM spermidine HCl
1 mM EDTA (pH 8.0)

Mix well, and place 0.5 μl of the reaction mixture in a tube containing 10 μl of 10 mM Tris · Cl (pH 8.0) and set aside to use in step 3.

2. Add 8 units (~1 μl) of bacteriophage T4 polynucleotide kinase to the reaction mixture. Mix well, and incubate for 45 minutes at 37°C. Place another 0.5-μl aliquot of the reaction in a second tube containing 10 μl of 10 mM Tris · Cl (pH 8.0) and set aside to use in step 3. Heat the remainder of the reaction for 10 minutes at 68°C to inactivate the bacteriophage T4 polynucleotide kinase.

3. Determine the efficiency of transfer of ^{32}P to the oligonucleotide and estimate its specific activity as follows (Stawinski et al. 1977; Narang et al. 1980):

   a. Cut a strip of cellulose impregnated with polyethyleneimine (Polygram CEL 300PEI; Brinkmann) about 5 cm wide and 15 cm long. With a soft-lead pencil, draw a fine line across the strip about 2.5 cm from one end, and make two small marks about one inch apart on the line.

   b. Spot 0.5 μl of each of the aliquots set aside in steps 1 and 2 at the marks, and place the strip vertically in a chromatography jar so that the radioactive samples are at the lower end of the strip. The bottom of the strip should dip into a petri dish filled to a level of approximately 0.5 cm with developing buffer (0.5 M ammonium bicarbonate). Cover the jar and allow the chromatograph to develop until the solvent front has migrated approximately 4–5 inches.

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c. Wrap the strip of polyethyleneimine-cellulose in Saran Wrap and autoradiograph, or cut the strip horizontally into thin (0.25-cm) sections and measure the amount of radioactivity in each section in a scintillation counter.

Oligonucleotides will remain at the origin, whereas ATP and inorganic phosphate will migrate in the same direction as the solvent (inorganic phosphate migrates slightly slower than the solvent front and ATP is approximately equidistant between the origin and the inorganic phosphate). Thus, the transfer of phosphate from [γ-^{32}P]ATP to the oligonucleotide will result in the appearance of radioactivity at the origin. By measuring the amount of radioactivity at the origin and on the total strip, the percentage of radiolabel transferred from [γ-^{32}P]ATP to the oligonucleotide can be calculated. The specific activity of the probe can then be determined on the basis of the molar quantities of oligonucleotide and [γ-^{32}P]ATP in the reaction. Under the conditions described above, approximately 50% of the radioactivity should be transferred to the oligonucleotide, resulting in a specific activity of approximately 2500 Ci/mmole.

The transfer of radiolabel to the oligonucleotide can also be monitored by adsorption to DE-81 filters. The oligonucleotide binds tightly to the positively charged filters, whereas unincorporated radiolabel is removed by repeated washing with a solution of sodium phosphate. See Appendix E for details.

4. *(Optional)* Remove unincorporated radiolabel from the oligonucleotide by one of the methods described in Chapter 11, pages 11.33–11.39.

Generally, this step is necessary only when background hybridization is a persistent problem. Under normal circumstances, the unpurified reaction mixture may be used as a probe.

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Screening Bacteriophage M13 Plaques by Hybridization to Radiolabeled Oligonucleotides

1. At the end of step 6e, page 15.65, transfer DNA from bacteriophage M13 plaques to nitrocellulose filters as follows:

   a. Transfer plates containing 100–500 plaques to a coldroom (4°C) for at least 30 minutes. Include at least one plate that contains plaques of the original unmutated recombinant bacteriophage M13. This serves as a negative control in the hybridization and washing steps.

   b. When the plates are thoroughly chilled, remove them from the cold-room and immediately lay a numbered, dry, nitrocellulose filter on the agar surface of each plate. Using a hypodermic needle (18 gauge), make a series of holes in each filter and the underlying agar. These holes will later serve to key the filters to the plates.

   c. Using blunt-ended forceps (e.g., Millipore forceps), carefully peel each filter from its plate. Spread out all of the filters (plaque side up) on a pad of paper towels. Wrap the plates in Saran Wrap, and store them at 4°C until they are needed.

   d. When the filters have dried (~30 minutes at room temperature), bake them for 1 hour at 80°C in a vacuum oven.

2. Transfer all of the filters to a heat-sealable plastic bag (e.g., Sears Seal-A-Meal), and add oligonucleotide prehybridization solution (~10 ml/82-mm filter). Seal the bag, and incubate the filters at 65°C for 1–2 hours.

   Oligonucleotide prehybridization solution

   6× SSC
   5× Denhardt's reagent (see Appendix B)
   0.1% SDS

3. Open the bag by cutting across a corner. Discard the prehybridization solution, and replace it with oligonucleotide hybridization mixture (~5 ml/82-mm filter). Reseal the bag, and incubate it at the appropriate temperature (see note below) for 4–6 hours.

   Oligonucleotide hybridization solution

   6× SSC
   5× Denhardt's reagent
   10^6–10^7 cpm/ml of radiolabeled oligonucleotide

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Hybridization with the radiolabeled oligonucleotide should be performed at a temperature 5–10°C below the T_m estimated from the following formula:

$$T_m = 4(G + C) + 2(A + T)$$

4. At the end of the hybridization period, cut off a corner of the plastic bag and pour the hybridization solution into a disposable plastic tube. Seal the tube, and store the solution at −20°C until it is needed for rescreening positive plaques (steps 10 and 16).

5. Quickly transfer the filters to a tray containing 200–300 ml of 6× SSC at room temperature. Cover the tray with Saran Wrap, and place it on a rotating shaker for 15 minutes. Replace the washing fluid every 5 minutes.

6. Quickly transfer the filters to a piece of Saran Wrap stretched on the bench. *Do not allow the filters to dry.* Cover the filters with another piece of Saran Wrap. Fold the edges of the two pieces of Saran Wrap together to form a tight seal. Apply adhesive dot labels marked with radioactive ink to the outside of the package and establish an autoradiograph by exposing the package of filters to X-ray film for 1–2 hours at −70°C, using an intensifying screen (see Appendix E).

   Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot (>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive warning tape to the pen, and store it in an appropriate place.

7. Compare the pattern of hybridization with the distribution of plaques. At this stage, it is normal to find that virtually every plaque hybridizes to the probe. Typically, however, some plaques hybridize more strongly than others, and these often turn out to be those that carry the desired mutation.

8. Transfer the filters to a plastic box containing 100–200 ml of 6× SSC that has been prewarmed to T_m − 10°C. Agitate the filters in the solution for 2 minutes, and then transfer them to a piece of Saran Wrap as described in step 6. Establish another autoradiograph. At this stage, it is often possible to identify two types of plaques: those whose radioactive signal has decreased in intensity and those that show no change in intensity.

   To minimize dissociation of perfect hybrids formed between the radioactive oligonucleotide and the mutagenized target sequence, do not wash the filters for more than 2 minutes.

9. Repeat the cycles of washing and autoradiography, increasing the temperature of the 6× SSC washing solution by 2–3°C in each cycle. The aim is to find a temperature that does not markedly affect perfect hybrids but causes dissociation of mismatched hybrids (such as those

formed between the mutagenic oligonucleotide and the original wild-type sequence). With practice, it is possible to eliminate some of the cycles of washing and autoradiography by monitoring the filters after each wash with a hand-held minimonitor.

10. Positively hybridizing plaques usually contain a mixture of both mutant and wild-type sequences. It is therefore *essential* to plaque-purify the bacteriophages from positively hybridizing plaques as follows:

    a. Touch the blunt end of a sterile, disposable wooden toothpick to the surface of a positively hybridizing plaque.

    b. Drop the toothpick into a sterile tube containing 1 ml of sterile TE (pH 7.6). Store the tube at room temperature for 10–15 minutes, shaking it from time to time to dislodge bacteriophage particles sticking to the toothpick.

    c. Make a series of tenfold dilutions of the bacteriophage suspension with TE (pH 7.6). Mix 10 μl of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions with 100-μl aliquots of an overnight culture of an appropriate strain of *E. coli* (e.g., TG1).

    d. Add 2.5 ml of 2 × YT top agar (melted and cooled to 45°C) to each culture, and plate the entire mixture on a single YT agar plate. Incubate the plate for 16 hours at 37°C to allow plaques to form.

    e. Rescreen the plaques with the radiolabeled oligonucleotide as described in steps 1–9. In this second round of screening, there is no need to increase the temperature of the washing solution in a stepwise fashion. Instead, the filters can be transferred directly from the washing solution at room temperature (step 5) to 6 × SSC prewarmed to the discriminatory temperature found empirically in step 9.

11. Pick two plaques from each of three independent putative mutants. Prepare single-stranded DNA from small-scale cultures infected with bacteriophages derived from each of these plaques, as described in Chapter 4, pages 4.29–4.30.

12. Carry out DNA sequencing, by the dideoxy-mediated chain-termination method (see Chapter 13), through the region containing the target sequence. Use either a universal sequencing primer or a custom-synthesized primer that binds 50–100 nucleotides upstream of the mutation site.

13. When the presence of the mutation has been confirmed, verify the sequence of the entire region of the target DNA cloned in the bacteriophage M13 vector to ensure that no adventitious mutations have been generated during propagation of the recombinant in bacteriophage M13. Often, this requires the synthesis of custom-designed sequencing primers that are complementary to segments of the target DNA spaced approximately 200 bp apart.

14. Isolate bacteriophage M13 replicative form DNA from a culture infected with plaque-purified recombinant bacteriophages (step 11) that carry the desired mutation and show no other changes in sequence in the target region. Methods to isolate and purify bacteriophage M13 replicative form DNA are given in Chapter 4.

15. Recover the mutated target sequence by digestion of bacteriophage M13 replicative form DNA with the appropriate restriction enzyme(s) and preparative gel electrophoresis. Reclone the target DNA in the desired vector.

16. Using a number of different restriction enzymes, digest aliquots of either a recombinant that carries the original (unmutagenized) target sequence or the recombinant (constructed in step 15) that carries mutagenized target sequence. Separate the resulting fragments by gel electrophoresis, and transfer them to a solid support (e.g., nitrocellulose filter or nylon membrane) as described in Chapter 9. Carry out Southern hybridization at $T_m - 10°C$, using the ^{32}P-labeled mutagenic oligonucleotide as probe. Wash the filter under the discriminatory conditions established in step 9 and autoradiograph. The final autoradiograph should show hybridization only to the relevant fragments of the mutagenized target DNA.

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Screening Bacterial Colonies by Hybridization to Radiolabeled Oligonucleotides

Colonies formed by bacteria transformed by mutagenized phagemids (see note to step 6, page 15.65) may be screened with ^{32}P-labeled oligonucleotide probes as described in detail in Chapter 11. However, because putative positive colonies almost certainly contain a mixture of wild-type and mutated molecules, it is necessary to carry out a second round of transformation and screening as outlined below. Note that simple restreaking of positive colonies does not generally yield pure populations of bacteria: The copy number of phagemids is so high that complete segregation of mutant from wild-type phagemids occurs only rarely.

- Transfer the transformed colonies to nitrocellulose filters as described in Chapter 1. Incubate the bacterial plates for a few hours at 37°C to allow the colonies to regrow, and then store the plates at 4°C until they are needed.
- Lyse the colonies transferred to the filter with alkali (see Chapter 1). Neutralize the filters, and then fix the liberated DNA in situ by baking for 1 hour at 80°C in a vacuum oven.
- Soak the filters in 2 × SSC, 0.1% SDS at 37°C. Using Kimwipes soaked in the same solution, gently rub the filters to remove all bacterial debris. Incubate the filters in fresh 2 × SSC, 0.1% SDS for 3 hours at 55°C.
- Carry out hybridization with the ^{32}P-labeled mutagenic oligonucleotide as described in steps 2–9 on pages 15.68–15.69.
- Identify colonies that hybridize preferentially with the mutagenic oligonucleotide. Set up a number of small-scale liquid cultures using individual colonies as inocula. Grow the cultures to saturation in the presence of the appropriate antibiotic.
- Isolate superhelical phagemid DNA from each of the cultures by one of the methods described in Chapter 1.
- Transform competent *E. coli* (strain MV1184) with each of the preparations of phagemid DNA.
- Prepare single-stranded phagemid DNA by superinfection of individual transformants as described in Chapter 4.
- Sequence the target DNA to verify the presence of the desired mutation and to confirm that no adventitious mutations have arisen.
- Recover the mutated fragment, reclone it into an appropriate vector, and confirm the presence of the mutation by Southern hybridization as described in steps 15 and 16 on page 15.71.

Note

An alternative method of obtaining pure mutant phagemids is to scrape the colonies transformed by the products of in vitro DNA synthesis (see note to

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step 6, page 15.65). Phagemid DNA is isolated from the pooled colonies and used to transform another batch of competent MV1184 cells. The resulting colonies, which will contain pure populations of either mutant or wild-type phagemids, are then screened by hybridization, using the mutagenic oligonucleotide as probe.

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OLIGONUCLEOTIDE-MEDIATED MUTAGENESIS BY SELECTION AGAINST TEMPLATE STRANDS THAT CONTAIN URACIL (KUNKEL METHOD)

This method (Kunkel 1985; Kunkel et al. 1987) uses single-stranded template DNAs in which a small number of residues have been replaced by uracil. These templates are generated in strains of *E. coli* carrying the *dut*$^-$ mutation, which leads to a deficiency in dUTPase (Konrad and Lehman 1975; Hochhauser and Weiss 1978). The intracellular pool of dUTP is greatly increased because of the cell's inability to convert dUTP to dUMP, and some of the dUTP is incorporated into DNA at sites normally occupied by thymine.

E. coli normally synthesizes an enzyme (uracil-*N*-glycosylase) that removes uracil residues that have been incorporated into DNA (Lindahl 1974; Duncan et al. 1978). However, in *ung*$^-$ strains, uracil is not removed, and a small fraction of the thymine residues in the bacterial DNA are therefore replaced by uracil. This proportion is increased in *dut*$^-$ *ung*$^-$ F′ strains of *E. coli* to the point where M13 bacteriophages grown in such strains contain between 20 and 30 uracil residues in their DNA. When these bacteriophages are used to infect an *ung*$^+$ strain of bacteria, the uracil is rapidly removed, generating sites that (1) block DNA synthesis and (2) are susceptible to cleavage by specific nucleases (for review, see Lindahl 1982). The destruction of the viral (+) strand results in a reduction of infectivity of approximately five orders of magnitude.

The Kunkel method of site-directed mutagenesis takes advantage of this strong selection against uracil-substituted DNA. The template DNA is first prepared by growth of an appropriate bacteriophage M13 recombinant in a strain of *E. coli* that is *dut*$^-$ *ung*$^-$ F′. The resulting uracil-containing single-stranded DNA is used as the template in a standard mutagenesis procedure to generate a heteroduplex molecule with uracil in the template strand and thymine in the strand synthesized in the in vitro reaction. Transformation of this DNA into an *ung*$^+$ strain results in destruction of the template strand, with consequent suppression of the production of wild-type bacteriophages. A large proportion (up to 80%) of the progeny bacteriophages are therefore derived from replication of the transfected uracil-free (−) strand. Because the synthesis of this strand is primed by the mutagenic oligonucleotide, many of the progeny bacteriophages carry the desired mutation (see Figure 15.8).

The protocol given below is a modification of the methods described by Kunkel (1985; Kunkel et al. 1987). In preparation for mutagenesis, a small fragment of DNA carrying the target sequence is cloned into an appropriate bacteriophage M13 vector (usually bacteriophage M13mp18 or M13mp19). Methods for cloning into bacteriophage M13 are described in Chapter 4.

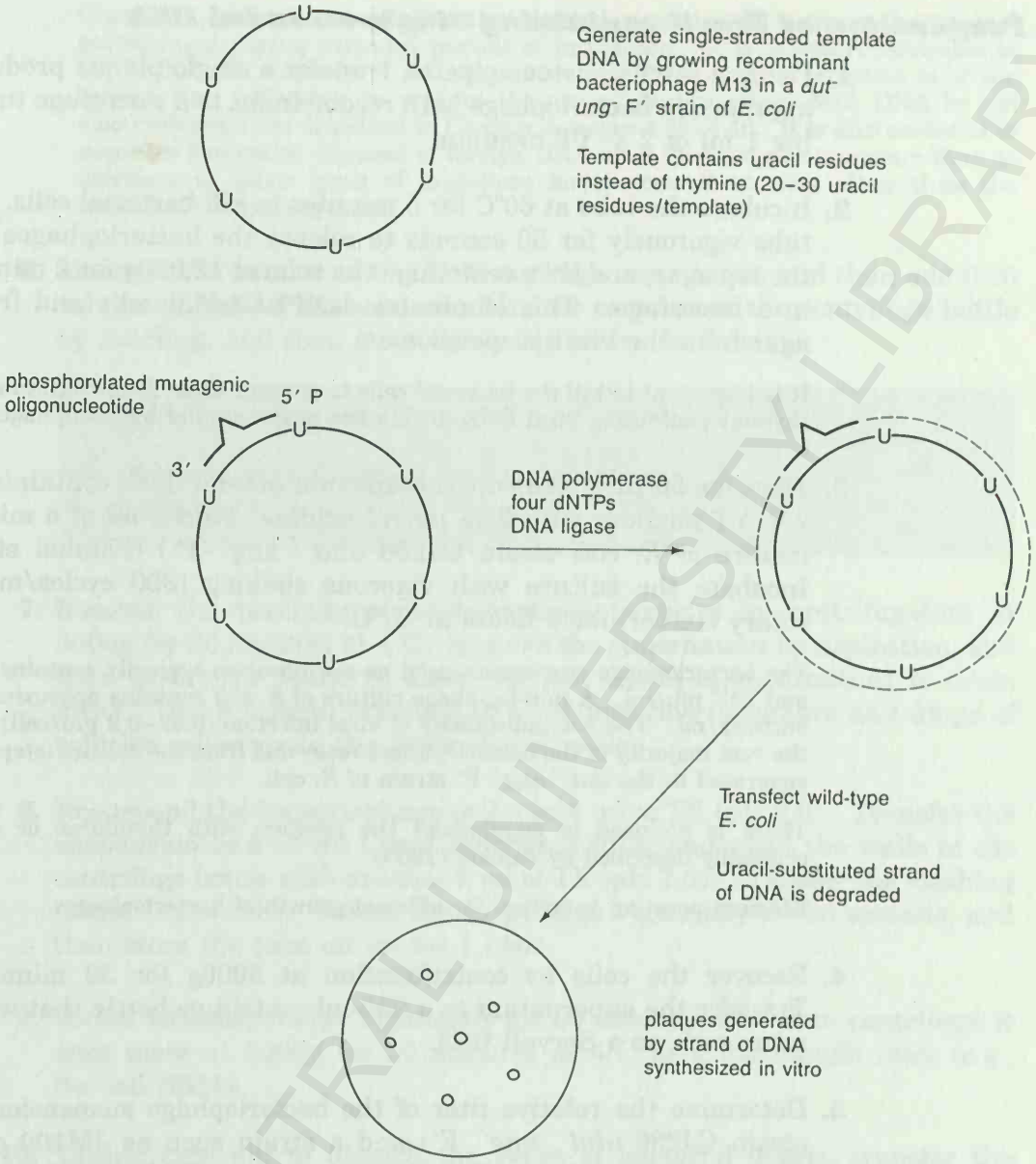
Generate single-stranded template DNA by growing recombinant bacteriophage M13 in a *dut⁻ ung⁻* F′ strain of *E. coli*
Template contains uracil residues instead of thymine (20–30 uracil residues/template)
phosphorylated mutagenic oligonucleotide
5′ P
3′
DNA polymerase
four dNTPs
DNA ligase
Transfect wild-type *E. coli*
Uracil-substituted strand of DNA is degraded
plaques generated by strand of DNA synthesized in vitro

FIGURE 15.8
Oligonucleotide-mediated, site-directed mutagenesis using the Kunkel method (see text for details).

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Preparation of Uracil-containing Single-stranded DNA

1. Using a sterile pasteur pipette, transfer a single plaque produced by the appropriate bacteriophage M13 recombinant to a microfuge tube containing 1 ml of 2× YT medium.

2. Incubate the tube at 60°C for 5 minutes to kill bacterial cells. Vortex the tube vigorously for 30 seconds to release the bacteriophages trapped in the top agar, and then centrifuge the tube at 12,000*g* for 2 minutes at 4°C in a microfuge. This eliminates dead bacterial cells and fragments of agar from the viral suspension.

   It is important to kill the bacterial cells to prevent them from continuing to produce thymine-containing viral DNA during the next round of bacteriophage growth.

3. Transfer 50 μl of the supernatant to a 500-ml flask containing 50 ml of 2× YT medium with 0.25 μg/ml uridine. Add 5 ml of a mid-log-phase culture of *E. coli* strain CJ236 (*dut*$^-$ *ung*$^-$ F′) (Kunkel et al. 1987). Incubate the culture with vigorous shaking (300 cycles/minute on a rotary shaker) for 6 hours at 37°C.

   The bacteriophage suspension used as an inoculum typically contains between 10^9 and 10^{10} pfu/ml. A mid-log-phase culture of *E. coli* contains approximately 5×10^8 bacteria/ml. The low multiplicity of viral infection (0.02–0.2 pfu/cell) ensures that the vast majority of the bacteriophages recovered from the culture (step 4) have been generated in the *dut*$^-$ *ung*$^-$ F′ strain of *E. coli*.

   There is no need to supplement the medium with thymidine or adenosine as originally described by Kunkel (1985).

   Efficient aeration is critical for efficient growth of bacteriophages.

4. Recover the cells by centrifugation at 5000*g* for 30 minutes at 4°C. Transfer the supernatant to a 250-ml centrifuge bottle that will fit into a rotor such as a Sorvall GS3.

5. Determine the relative titer of the bacteriophage suspension on *E. coli* strain CJ236 (*dut*$^-$ *ung*$^-$ F′) and a strain such as JM109 or TG1 (see Chapter 4, page 4.14). The titer on strain CJ236 should be four to five orders of magnitude greater than on the other strain used.

   To save time, most workers proceed with the purification of bacteriophage particles before the results of the titration are available. If the purification is postponed, the crude bacteriophage suspension should be stored in ice.

   The yield of bacteriophages varies from recombinant to recombinant. Typically, the titer of virus particles in the supernatant, determined on a *dut*$^-$ *ung*$^-$ F′ strain (CJ236), is 5×10^{10}–1×10^{11} pfu/ml. However, poorly growing recombinants may attain titers of only 1×10^{10}–2×10^{10} pfu/ml. If the yield of single-stranded DNA is inadequate, two possible remedies are:
   - Determine the titer of the bacteriophage stock used to infect the *dut*$^-$ *ung*$^-$ F′ strain of *E. coli*. Adjust the volume of the inoculum so as to achieve a multiplicity of infection of 0.1 pfu/bacterial cell. Grow the infected culture for 6 hours as described.
   - Incubate the infected cultures for 12 hours rather than 6 hours. As discussed in

Chapter 4, there is a possibility that deleted variants will outgrow the original recombinant during extended periods of incubation. It is therefore advisable to verify that the majority of the single-stranded DNA used as template is of the correct size. Methods to analyze the size of bacteriophage M13 DNA by gel electrophoresis are described in Chapter 4, pages 4.39–4.40. It is also essential to sequence the entire segment of foreign DNA after mutagenesis to ensure that no deletions or other types of mutations have occurred at sites other than the immediate target sequence.

6. Measure the volume of the bacteriophage suspension, and then add 0.25 volume of NaCl/PEG solution. Mix the contents of the centrifuge bottle by swirling, and then store the bottle on ice for 1 hour.

NaCl/PEG solution

15% w/v polyethylene glycol (PEG 8000)
2.5 M NaCl

7. Recover the precipitated bacteriophage particles by centrifugation at 5000*g* for 20 minutes at 4°C. Remove the supernatant by aspiration, and then invert the bottle to allow the last traces of supernatant to drain away. Use a pipette attached to a vacuum line to remove any drops of solution adhering to the walls of the bottle.

8. Resuspend the bacteriophage pellet in 4 ml of TE (pH 7.6). Transfer the suspension to a 15-ml Corex centrifuge tube, and wash the walls of the centrifuge bottle with another 2 ml of TE (pH 7.6). Transfer the washing to the Corex tube. Vortex the suspension vigorously for 30 seconds, and then store the tube on ice for 1 hour.

9. Vortex the suspension vigorously for 30 seconds, and then centrifuge it once more at 5000*g* for 20 minutes at 4°C in a fixed-angle rotor (e.g., Sorvall SS34).

10. Taking care not to disturb the pellet of bacterial debris, transfer the supernatant to a polypropylene tube. Extract the suspension twice with phenol equilibrated to pH 8.0 (see Appendix B) and once with phenol: chloroform. Separate the phases by centrifugation at 4000*g* for 5 minutes at room temperature. Avoid transferring material from the interface.

11. Transfer the aqueous phase from the final extraction to a glass centrifuge tube (e.g., a 30-ml Corex tube). Measure the volume of the solution, and add 0.1 volume of 3 M sodium acetate (pH 5.2), followed by 2 volumes of ethanol at 0°C. Mix the contents of the tube thoroughly, and then store the tube on ice for 30 minutes.

12. Recover the DNA by centrifugation at 5000*g* for 20 minutes at 4°C. Carefully remove the supernatant. Add 10 ml of 70% ethanol at room temperature, vortex briefly, and recentrifuge.

13. Remove the supernatant, and store the inverted tube at room temperature until the last traces of ethanol have evaporated. Redissolve the DNA in 200 μl of TE (pH 7.6).

14. Measure the DNA spectrophotometrically at 260 nm (1 $OD_{260} = 40$ μg/ml) (see Appendix E). Analyze the size of an aliquot of the DNA (0.5 μg) by gel electrophoresis, using as marker single-stranded DNA of the original bacteriophage M13 recombinant (step 1).

15. Carry out oligonucleotide-mediated, site-directed mutagenesis as described on page 15.63 (beginning at step 2), with the following modifications:

- Use as primers either the phosphorylated mutagenic primer alone or the phosphorylated mutagenic primer and a *phosphorylated* universal sequencing primer. Do not use a nonphosphorylated sequencing primer in the polymerization/extension reaction because this cannot be ligated to the 3′ terminus of the newly synthesized strand. The efficiency of transfection in *ung*$^+$ bacteria of bacteriophage M13 DNA that contains uracil in one strand is greatly increased if the thymine-containing strand is covalently closed. This can only happen if the priming oligonucleotide(s) is phosphorylated. Before use in site-directed mutagenesis, the mutagenic oligonucleotide should be purified by Sep-Pak C_{18} column chromatography, as described in Chapter 11, page 11.39, to remove salts and other impurities. However, it is generally not necessary to purify the oligonucleotide by polyacrylamide gel electrophoresis unless the oligonucleotide is more than 30 nucleotides in length or is to be used for "loop-in" or "loop-out" mutagenesis.

- If a single phosphorylated mutagenic oligonucleotide is used as a primer, bacteriophage T4 DNA polymerase (Nossal 1974; Kunkel 1985) or Sequenase (Schena 1989) should be used in the polymerization/extension reaction. Unlike the Klenow fragment of *E. coli* DNA polymerase I, neither of these enzymes is able to displace the mutagenic oligonucleotide from its template. Use dNTPs of the highest quality to minimize the possibility that contaminating dUTP might be incorporated into the newly synthesized strand of DNA. The concentrated dNTP solutions sold by Pharmacia have worked well in our hands.

  When using bacteriophage T4 DNA polymerase or Sequenase, the extension reaction should be incubated for 5 minutes at 0°C, 5 minutes at room temperature, and then for 2 hours at 37°C. The low temperature optimizes initiation of DNA synthesis from the 3′ terminus, and subsequent incubation at 37°C improves the efficiency of the extension reaction. In addition, the concentration of each of the four dNTPs in the reaction should be increased to 500 μM when using these polymerases. This increases the efficiency of the extension reaction and suppresses the strong 3′ exonuclease activity of bacteriophage T4 DNA polymerase.

- Use 1-μl and 5-μl aliquots of the undiluted polymerization/extension reaction to transfect competent cultures of *E. coli* strain TG1.

- Because growth of unmutated bacteriophages is suppressed with high efficiency, there is often no need to screen plaques by hybridization. Instead, single-stranded DNA can be prepared from a number of well-isolated plaques and analyzed directly by DNA sequencing.

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SOLVING PROBLEMS THAT ARISE IN OLIGONUCLEOTIDE-MEDIATED, SITE-DIRECTED MUTAGENESIS

Below are listed some problems that can arise in oligonucleotide-mediated, site-directed mutagenesis, along with suggested solutions for these problems.

1. Plaques or colonies are obtained that hybridize specifically to the mutagenic oligonucleotide, but the target sequence does not show the desired mutation.

   - Purify the mutagenic oligonucleotide by polyacrylamide gel electrophoresis.
   - Test the mutagenic oligonucleotide for its ability to prime DNA synthesis at the appropriate location on the single-stranded template DNA (Zoller and Smith 1987). Use universal sequencing primer as a positive control.

     If priming occurs from more than one site, synthesize a longer mutagenic oligonucleotide that binds specifically to the template DNA. Use a shorter oligonucleotide as a hybridization probe to screen for the desired mutant.

     If priming by the mutagenic oligonucleotide is inefficient, purify the oligonucleotide by polyacrylamide gel electrophoresis or resynthesize the oligonucleotide or do both.

2. No plaques or colonies are obtained that hybridize specifically to the mutagenic oligonucleotide.

   - Test the mutagenic oligonucleotide for its ability to prime DNA synthesis at the appropriate location on the single-stranded template DNA (Zoller and Smith 1987). Use universal sequencing primer as a positive control.
   - Check whether the mutagenic oligonucleotide or the single-stranded template DNA has a high capacity to form secondary structures. If so, synthesize a longer mutagenic oligonucleotide that extends beyond the problem regions. Use a shorter oligonucleotide as a hybridization probe to screen for the desired mutant.

3. All plaques or colonies hybridize to the oligonucleotide probe.

   - Check that there is no region of extensive homology between the oligonucleotide probe and ectopic regions of the vector or the target DNA.
   - Increase the stringency of washing. Remove bacterial debris from the filters before hybridization by wiping the filters with Kimwipes soaked in warm 2× SSC, 0.1% SDS.
   - Purify the mutagenic oligonucleotide by polyacrylamide gel electrophoresis. If the problem persists, resynthesize the oligonucleotide. Consider synthesizing a shorter oligonucleotide.

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Using Mutagenesis to Study Proteins

Until a few years ago, only two methods were available to study directly the relationship between the structure and function of a protein: (1) chemical modification of the side chains of the amino acids that form the primary sequence of the protein and (2) X-ray diffraction of protein crystals. Although both of these methods have yielded much information, they can be used only with proteins that are available in large quantity and are of high purity. A less direct type of analysis may be used if naturally occurring mutations can be identified that map in the gene coding for the protein of interest and that generate an observable phenotype. The mutant genes can then be cloned, and their DNA sequences can be compared to that of the wild-type allele. This approach has been invaluable in identifying mutations that affect the function of proteins such as the human LDL receptor (Davis et al. 1986) and factor VIII (Gitschier et al. 1988). However, this method also has its own set of restrictions: The mutations are sometimes confined to specific domains of the protein, and it is often difficult to isolate a sufficient number of independent mutants to allow definitive conclusions to be drawn about the structure of the protein.

These constraints can be partially circumvented by using site-directed mutagenesis to introduce mutations at predetermined sites in a cloned cDNA and then expressing the altered gene in an appropriate host-cell/vector system. By comparing the properties of the mutant and wild-type forms of the protein, it is often possible to identify domains or individual amino acid residues that are essential for the structural integrity and/or biological function of the protein. Because of the rapid advances that have occurred in recent years in both site-directed mutagenesis and expression of cloned genes, this method, which is sometimes called "reversed genetics," is often the first approach used by molecular biologists to analyze the relationship between a protein's structure and its function. The major problem with reversed genetics, however, is how to distinguish mutations that affect local structures from those that have profoundly deleterious effects on the folding or stability of the entire protein. Consider a typical experiment in which a number of point mutations have been generated at various sites in a gene coding for an enzyme. When the activities of these mutants are assayed, some of them show a reduction in catalytic function and others do not. In the absence of any other data, it is not possible to draw firm conclusions about the structure of the enzyme from this result. There is no way to know whether the substitution of one amino acid for another has affected only the structure and function of the active site or whether it has had more global effects. The problem would remain even if the three-dimensional shape of the wild-type enzyme were known. No algorithms have been yet devised that accurately predict the perturbations in protein structure caused by substitution, addition, or deletion of amino acid residues.

These difficulties can be alleviated by developing independent assays for the folding of the protein of interest. Such assays typically include:

- *The ability of the protein to react with mono- or polyclonal antibodies that are specific for native or unfolded epitopes.* In the example given above, if a catalytically inactive mutant retains its full ability to react with antisera specific for native epitopes, it is reasonable to conclude that the effects of the mutation have not spread throughout the entire structure of the enzyme. Conversely, if the mutant reacts with several antisera that recognize only denatured or unfolded forms of the protein, the effects of the mutation are almost certainly global.

- *The retention of other functions by the protein.* For example, if a catalytically inactive mutant retains its ability to bind specific cofactors or other ligands such as ATP, at least a part of its structure must be folded in the normal fashion.

- *The formation of correct quaternary structures.* Many proteins consist of two or more polypeptide subunits that are held together by specific hydrophobic or hydrophilic contacts. The ability to form these contacts and to generate quaternary structures with the correct stoichiometry and stability is a strong indication that the individual polypeptides are folded in a normal fashion.

- *The resistance of the protein to digestion with proteases.* Correctly folded proteins are generally more resistant to digestion with proteases such as trypsin and proteinase K than are malfolded proteins. By comparing the ability of mutant and wild-type forms of the target protein to resist digestion by different concentrations of protease, it is often possible to determine whether or not the mutant protein is severely malfolded.

- *In those cases where sufficient material can be obtained, the ability of the mutant protein to display normal biophysical characteristics (e.g., circular dichroism spectra, sedimentation velocity, and thermal stability).*

In addition, many difficulties can be avoided by choosing carefully the type and location of mutations before they are constructed by site-directed mutagenesis. When designing mutations in proteins whose structures are unknown, the following guidelines may be helpful.

1. Analyze the amino acid sequence carefully by several computer programs to determine which regions of the protein are likely to form turns, loops, β sheets, and α helices. The criteria used to predict secondary structure vary from program to program, and the final predictions are never in exact agreement. However, whenever different programs agree on the structure of a particular region of the protein, there is a good chance that the structure will, in fact, exist. When planning sites for mutagenesis, remember that (a) amino acids located in hydrophobic regions are likely to lie in the core of the protein; (b) α helices and β-sheet elements are more likely to serve as scaffolds than to be directly involved in catalysis or ligand binding; and (c) loops, turns, and clusters of charged residues are likely to lie on the surface of the protein where they may be involved in protein–protein interaction or ligand binding.

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2. Search the data banks for proteins with homologous amino acid sequences. Residues that are highly conserved between proteins of different function are more likely to be involved in forming common structural motifs than in highly specific and private activities such as catalysis or ligand binding.

3. Use a computer program to analyze whether the amino acid sequence contains internal repetitions. These indicate that the gene coding for the protein has evolved by one or more rounds of duplication followed by mutagenic drift. Residues that are conserved between different repetitive elements are more likely to be involved in forming common structural motifs than in catalysis or ligand binding.

4. When planning to make deletion mutants, avoid the temptation to remove the DNA sequences that lie between naturally occurring restriction sites. Although such deletions are easy to make, their borders are unlikely to lie at sensible positions within the coding sequence and the resulting proteins are almost certain to be malfolded.

5. If the positions of introns and exons have been established by analysis of the corresponding segment of genomic DNA, consider the possibility of creating a set of deletion mutants that lack particular exons or combinations of exons. This approach has been particularly useful in analyzing the structure and function of proteins that have evolved by exon shuffling (see, e.g., Gething et al. 1988). Because individual exons often encode independently folding polypeptide domains, there is a good chance that precise removal of a particular exon will not prejudice the folding of the remainder of the protein.

6. When constructing chimeras between nonhomologous proteins, try to arrange for the junctions between the two coding regions to lie at the borders of predicted structural domains rather than within them. For example, the ectodomains, transmembrane regions, or cytoplasmic tails of different transmembrane receptors should be exchanged at residues that are predicted to lie close to the appropriate transmembrane region. However, if the two proteins are homologous in sequence and function, the junctions should be located in segments of amino acid sequence that are identical or nearly so.

7. Avoid changing proline residues (all proteins) or cysteine residues (secretory or cell-surface proteins). These amino acids are usually involved in forming and maintaining essential structural motifs. Proline is the residue that is frequently used to terminate α-helical regions, whereas pairs of cysteine residues form the stabilizing disulfide bonds that are a hallmark of many secretory and cell-surface proteins.

8. When designing point mutations in secretory or transmembrane proteins, avoid altering asparagine or serine/threonine residues that lie within potential sites for *N*-linked glycosylation (Asn-X-Ser/Thr, where X is any amino acid other than proline). Mannose-rich oligosaccharides that are added posttranslationally to the asparagine residues in such consensus sequences are in some cases involved in assisting the nascent polypeptide to fold (Gallagher et al. 1988).

9. When the three-dimensional structure of the target protein is not known, functional domains can sometimes be identified by the insertion at random sites of a synthetic hexameric linker that does not change the reading frame but adds two amino acids to the protein (Stone et al. 1984; Barany 1985a,b, 1987, 1988). The insertion of just two amino acids is claimed to be less disruptive to protein structure than other types of insertional mutagenesis in which insertion of a linker results in the addition of three or four amino acids. Whatever the size of the insertion, however, it is essential to develop independent methods (see above) to distinguish between global and localized effects of the mutation.

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INSERTION OF HEXAMERIC LINKERS INTO PROTEIN-CODING SEQUENCES

The method given below, which is an elaboration of the work of Boeke (1981), is based on protocols published by Barany (1985a,b, 1987, 1988). Synthetic hexameric linkers are inserted quasi-randomly into the gene by targeting insertion to sites for frequently cutting restriction enzymes. The sequence of the hexameric linker is chosen so that no termination codons are inserted. A selectable marker (Vieira and Messing 1982) is used to identify mutants that have incorporated the linker. Although straightforward, the approach is limited to insertions at preexisting restriction enzyme sites. Nevertheless, this approach can be very useful in the initial determination of functional domains of a protein. The method involves the following steps:

- Cloning the target into a suitable plasmid (e.g., pUC18/pUC19 or pUC118/pUC119)
- Linearization of the plasmid DNA by partial digestion with a restriction enzyme that cleaves frequently within the target sequence and generates cohesive termini
- Ligation of a single-stranded hexameric linker to the cohesive termini
- Removal of excess linkers by cleavage with the appropriate restriction enzyme
- Ligation of a fragment of DNA carrying a selectable marker (usually *kan*^{r}) to the cohesive termini of the linkers
- Selection of transformed bacteria that are resistant to kanamycin and ampicillin
- Removal of the fragment of DNA carrying the selectable marker by cleavage with the appropriate restriction enzyme
- Recircularization of the linear DNA molecule lacking the *kan*^{r} gene
- Transformation of bacteria with the recircularized plasmid
- Screening of transformed colonies for those that carry plasmids containing a novel restriction site within the target sequence

This method is therefore a variation of the technique described on pages 15.32–15.50 that is used to construct linker-scanning mutations. A preassembled kit can be purchased from Pharmacia (TAB™ mutagenesis system) that contains the materials required for this type of mutagenesis.

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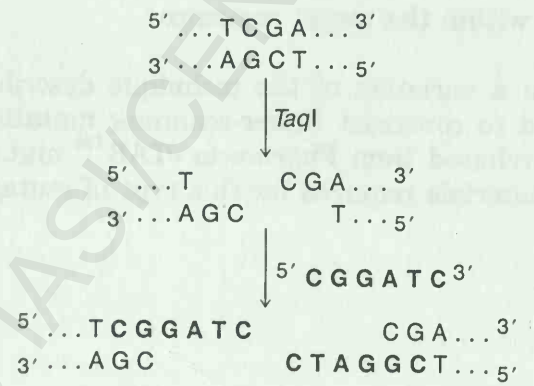
Designing the Hexameric Linker

Before starting hexameric linker mutagenesis, it is necessary to decide (1) which restriction enzyme will be used to linearize the plasmid DNA and (2) the restriction site that will be inserted into the target DNA as part of the hexameric linker. (*Note:* The amino acids that will be inserted in the mutant protein depend on the reading frame and on the sequence of the linkers.) The following factors should be taken into consideration:

- *The frequency and distribution of the restriction sites within the target DNA.* Choose a restriction enzyme to linearize the plasmid that cleaves the target DNA at many different places, preferably generating a protruding terminus two nucleotides in length (e.g., *Taq*I $^{5'}..T^{\downarrow}CGA..^{3'}$; *Hha*I $^{5'}..GCG^{\downarrow}C..^{3'}$). (*Note:* If there is no other choice, restriction enzymes that generate blunt termini can be used [Barany 1988]. However, the efficiency of mutagenesis is then markedly reduced, presumably because double-stranded hexameric linkers must be used in place of single-stranded linkers.)
- *The restriction site that is to be inserted as part of the hexameric linker should not occur at any other place in the target DNA or the plasmid vector.*
- *The choice of restriction sites that can be inserted is dictated by the nature of the termini of the linearized plasmid.* For example, the 5′ C residue created by cleavage with *Taq*I would become the last nucleotide of the restriction site that is to be created by insertion of the hexameric linker. Similarly the 3′ G residue created by cleavage with *Hha*I would become the first nucleotide of the new restriction site.
- *A fragment of DNA must be available that carries the* kan^r *gene.* Each end of this DNA must carry the same restriction site that is chosen for use in the hexameric linker. A number of cassettes carrying the *kan*^{r} gene are now available (see Barany 1987, 1988).

When decisions have been made about restriction sites and restriction enzymes, design the hexameric linkers. For example, to create a *Bam*HI site at a *Taq*I terminus, the sequence of the hexameric linker should be:



To create an *Eco*RI site at a *Hha*I terminus, the sequence of the hexameric linker should be:

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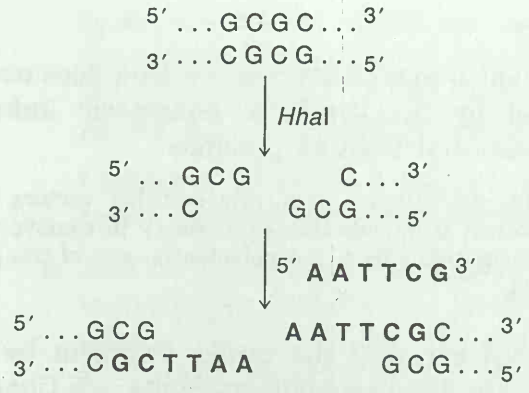

When the appropriate hexameric linker has been synthesized, proceed as described on pages 15.88–15.94.

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Insertion of Hexameric Linkers

1. Clone the target fragment into a plasmid vector that does not contain the restriction site carried by the synthetic hexameric linker and that contains as little nonessential DNA as possible.

   pUC18/pUC19 or pUC118/pUC119 are generally suitable vectors for hexameric linker mutagenesis. However, it is sometimes necessary to remove the restriction site carried by the hexameric linker from the polycloning site of the plasmid before the target DNA is inserted.

2. Purify the plasmid DNA carrying the target fragment by equilibrium centrifugation in CsCl–ethidium bromide gradients (see Chapter 1, pages 1.42–1.45). Approximately 40 μg of closed circular DNA will be required.

   The DNA should be freshly purified to minimize the proportion of linear and relaxed circular forms of the plasmid in the preparation.

3. While the CsCl–ethidium bromide gradients are being centrifuged, digest approximately 10 μg of an appropriate plasmid containing the *kan*^{r} gene with a restriction enzyme that will generate a fragment of DNA carrying the *kan*^{r} gene. Purify the fragment by preparative agarose gel electrophoresis using one of the methods described in Chapter 6. Redissolve the purified fragment in TE (pH 7.6) at a concentration of approximately 250 μg/ml. Two to three micrograms of the fragment will be sufficient for several experiments.

4. Linearize the plasmid carrying the target DNA with a restriction enzyme that cleaves that target DNA many times and generates cohesive termini. This is best achieved by carrying out the digestion in the presence of ethidium bromide, a drug that intercalates into double-stranded DNA and slows down the rate of cleavage by most, but not all, restriction enzymes (Österlund et al. 1982). Because closed circular double-stranded DNA binds less ethidium bromide than linear DNA, the first cleavage by a restriction enzyme is not inhibited as efficiently as the subsequent cleavages. Thus, at the beginning of the digestion, there is a rapid accumulation of full-length linear molecules. Ethidium bromide is also thought to suppress the tendency of restriction enzymes to cleave preferentially at certain sites. Because different restriction enzymes (and even different batches of the same restriction enzyme) are inhibited to varying extents by ethidium bromide, it is necessary to carry out a pilot experiment to determine the conditions that generate a reasonable yield of full-length linear molecules.

   Pilot experiment

   a. Set up three tubes, each containing 5 μg of closed circular plasmid DNA in a total volume of 44 μl of the appropriate 1× restriction enzyme buffer. Add 1 μl of a solution of ethidium bromide (1 mg/ml in water) to the first tube, and immediately mix the contents of the tube by vortexing. Add 2 μl and 3 μl of the ethidium bromide solution to the second and third tubes, respectively. After the ethidium

bromide has been added, use 1 × restriction enzyme buffer to adjust the volume of the solution in each tube to 49 μl. The concentrations of ethidium bromide in the three digestion mixtures are therefore 20 μg/ml, 40 μg/ml, and 60 μg/ml, respectively.

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Follow precautions detailed in step 1h, page 15.9.

b. Add 10 units of the appropriate restriction enzyme to each tube, and immediately transfer the three tubes to a water bath set at 37°C. After 0, 5, 10, 20, 30, 45, and 60 minutes of incubation, transfer aliquots (5 μl) from each of the three tubes to fresh microfuge tubes containing 15 μl of 5 mM EDTA (pH 8.0). Heat each sample for 10 minutes at 68°C. Store the tubes containing the aliquots on ice until all of the samples have been collected.

c. Analyze the DNA in each sample by electrophoresis through a 0.8% agarose gel cast and run in 0.5 × TBE (see Appendix B) containing ethidium bromide (0.5 μg/ml). As markers, use plasmid DNA that has been linearized by digestion with a restriction enzyme that cleaves at only one site. Under these conditions of electrophoresis, closed circular DNA migrates slightly faster than linear DNA and considerably faster than relaxed circular DNA.

d. Examine the gel by ultraviolet illumination, and determine the conditions that give the maximum yield of full-length linear molecules. Usually not more than 33% of the original closed circular plasmid DNA is converted to full-length linear molecules.

Caution: Ultraviolet radiation is dangerous, particularly to the eyes. Follow precautions detailed in step 1i, page 15.9.

e. Set up a large-scale digestion containing 20 μg of closed circular DNA. Increase the volume of all of the components of the reaction proportionally, and incubate the reaction for the time required to achieve maximal conversion of closed circular DNA to full-length linear molecules.

5. Purify the full-length linear DNA by preparative agarose gel electrophoresis using one of the methods described in Chapter 6. Redissolve the DNA in TE (pH 7.6) at a concentration of 250 μg/ml. Approximately 1–2 μg of full-length linear DNA will be required to complete the remainder of the procedure.

6. a. *Ligation of single-stranded hexameric linkers to linear plasmid DNA carrying protruding 5′ termini and removal of excess linkers*

i. Phosphorylate approximately 10 μg of hexameric linkers with bacteriophage T4 polynucleotide kinase as described on page 15.63. The total volume of the reaction should not exceed 10 μl.

ii. Ligate the phosphorylated linkers to the linear plasmid DNA as follows:

Mix:

linear plasmid DNA	0.5 μg
phosphorylated hexameric linkers (1 μg/μl)	2 μl
10 × ligase buffer	1 μl
H_2O to 9 μl	

Add 2 Weiss units of bacteriophage T4 DNA ligase and 10 mM ATP to a final concentration of 1 mM. Incubate the mixture for 12–16 hours at 4°C, and then add another aliquot (2 Weiss units) of bacteriophage T4 DNA ligase and continue incubation for a further 1 hour at 16°C.

10 × Ligase buffer

0.2 M Tris · Cl (pH 7.6)
50 mM $MgCl_2$
50 mM dithiothreitol

For definition of Weiss unit, see page 15.30.

iii. Heat the ligation mixture to 68°C for 10 minutes to inactivate the bacteriophage T4 DNA ligase.

iv. Add:

appropriate 10 × restriction enzyme buffer	5 μl
H_2O	35 μl
restriction enzyme that recognizes the site created by ligation of the hexameric linkers to the linearized plasmid DNA	20 units

v. Incubate the mixture at the appropriate temperature for 1–2 hours.

vi. Purify the DNA by extraction with phenol:chloroform, and recover the DNA from the aqueous phase by precipitation with 0.5 volume of ammonium acetate and 2.5 volumes of ethanol. Store the tube for 10 minutes on ice, and recover the DNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Remove the supernatant and wash the pellet of DNA carefully with 70% ethanol. Allow the pellet to dry at room temperature, and then redissolve it in 20 μl of TE (pH 7.6).

vii. Proceed to step 7.

b. *Ligation of single-stranded hexameric linkers to linear plasmid DNA carrying protruding 3′ termini and removal of excess linkers*

i. Ligate the *unphosphorylated* hexameric linker to the linear plasmid DNA as described for phosphorylated linkers in step 6a, ii.

ii. Heat the ligation mixture to 68°C for 10 minutes to inactivate the bacteriophage T4 DNA ligase.

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iii. Add:

10 × bacteriophage T4 polynucleotide kinase buffer	2 μl
10 mM ATP	2 μl
H_2O	6 μl
bacteriophage T4 polynucleotide kinase	10 units

Incubate the reaction at 37°C for 30 minutes, and then heat the mixture to 68°C for 15 minutes to inactivate the bacteriophage T4 polynucleotide kinase.

10 × Bacteriophage T4 polynucleotide kinase buffer

0.5 M Tris · Cl (pH 7.6)
0.1 M $MgCl_2$
50 mM dithiothreitol
1 mM spermidine HCl
1 mM EDTA (pH 8.0)

iv. Purify the DNA by extraction with phenol:chloroform, and recover the DNA from the aqueous phase by precipitation with 0.5 volume of ammonium acetate and 2.5 volumes of ethanol. Store the tube for 10 minutes on ice, and recover the DNA by centrifugation at 12,000*g* for 15 minutes at 4°C in a microfuge. Remove the supernatant and wash the pellet of DNA carefully with 70% ethanol. Allow the pellet to dry at room temperature, and then redissolve it in 20 μl of TE (pH 7.6).

v. Proceed to step 7.

7. Add:

H_2O	18 μl
10 × ligase buffer	5 μl
purified fragment of DNA that carries the *kan*^{r} gene (step 3) (0.5 μg)	2 μl

Add 5 Weiss units of bacteriophage T4 DNA ligase and 10 mM ATP to a final concentration of 1 mM. Incubate the reaction for 6–8 hours at 16°C.

In a separate tube, set up a control containing all of the components of the ligation mixture except the linearized plasmid DNA to which hexameric linkers have been added and incubate as above.

8. Use 10-μl aliquots of the control and test ligations to transform competent *E. coli* of an appropriate strain (e.g., DH1 or DH5) to resistance to kanamycin and ampicillin. Select transformants on LB agar plates containing kanamycin and ampicillin (or carbenicillin), each at a concentration of 100 μg/ml. If the experiment has gone well, the test ligation should generate approximately tenfold more colonies than the control ligation.

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Ampicillin or carbenicillin is included to select against plasmids in which the DNA fragment carrying the *kan*^{r} gene has been inserted into the *amp*^{r} gene rather than into the target sequence.

9. Pick 36 independent transformants, and establish small-scale (2-ml) cultures in LB medium containing ampicillin and kanamycin, each at a concentration of 100 μg/ml. After the cultures have reached saturation, isolate the plasmid DNA using one of the methods described in Chapter 1. Analyze the DNAs by digestion with the appropriate restriction enzymes and gel electrophoresis to obtain an estimate of the proportion of clones that carry a linker in the target region.

10. When the results of this preliminary screen are available, recover the Kan^r, Amp^r colonies from the original transformation plates by adding to each plate approximately 5 ml of LB medium containing ampicillin and kanamycin, each at a concentration of 100 μg/ml. Use a bent glass rod and a pasteur pipette to scrape and squirt the colonies from the surfaces of the plates. Pool the bacterial suspension obtained from each of the plates, and recover the bacterial cells by centrifugation at 5000*g* for 10 minutes at 4°C. Remove the supernatant medium.

11. Isolate the closed circular plasmid DNA from the pooled colonies by one of the small-scale methods described in Chapter 1.

12. Recover the target DNA fragments containing the inserted *kan*^{r} gene by digesting the preparation of pooled plasmid DNAs with the appropriate restriction enzyme (or combination of restriction enzymes).

    Insertion of the fragment carrying the *kan*^{r} gene increases the size of the target fragment by approximately 1.4 kb.

13. Purify the target fragments carrying the *kan*^{r} gene by electrophoresis through a 0.7% agarose gel.

14. Ligate the purified target fragments to an appropriately prepared plasmid vector (e.g., pUC118/pUC119). Because the efficiency with which the target fragments will ligate to the plasmid vector is unknown, small pilot reactions should be set up to establish the optimum ratio of DNA fragments in the reaction. For example, dissolve 200 ng of plasmid DNA and 0, 100, 250, or 500 ng of the target fragments in 12.5 μl of TE (pH 7.6). To each mixture, add 1.5 μl of 10 $\times$ ligase buffer, followed by 0.5 Weiss unit of bacteriophage T4 DNA ligase. Incubate the reactions for at least 4 hours at 16°C. Use small aliquots (2 μl of each mixture) to transform competent *E. coli* (strain DH1 or DH5) simultaneously to resistance to kanamycin and ampicillin (i.e., the bacterial plates used to select transformants should contain both kanamycin and ampicillin, each at a concentration of 100 μg/ml). Store the remainders of the ligation mixtures at -20°C.

15. Recover the Kan^r, Amp^r colonies from the original transformation plates by adding to each plate approximately 5 ml of LB medium containing

ampicillin and kanamycin, each at a concentration of 100 μg/ml. Use a bent glass rod and a pasteur pipette to scrape and squirt the colonies from the surfaces of the plates. Pool the bacterial suspension obtained from each of the plates, and recover the bacterial cells by centrifugation at 5000*g* for 10 minutes at 4°C. Remove the supernatant medium.

16. Isolate the closed circular plasmid DNA from the pooled colonies by one of the small-scale methods described in Chapter 1.

17. To remove the fragment carrying the *kan*^{r} gene from the plasmids containing the synthetic linker, digest the preparation of pooled plasmid DNAs with a restriction enzyme that recognizes the site created by ligation of the hexameric linkers.

18. When the restriction digest is complete, dilute the reaction mixture with 1 × ligase buffer until the concentration of the plasmid DNA is < 3 μg/ml. Add approximately 5 Weiss units of bacteriophage T4 DNA ligase per 100 μl of reaction mixture. Incubate the reaction for 4 hours at 16°C.

1 × Ligase buffer

20 mM Tris · Cl (pH 7.6)
5 mM $MgCl_2$
5 mM dithiothreitol
1 mM ATP

19. Concentrate the DNA by extracting the ligation mixture twice with 1-butanol (see Appendix E). Add 0.2 volume of 10 M ammonium acetate, and precipitate the DNA with 2.5 volumes of ice-cold ethanol. Store the tube in an ice bath for 30 minutes.

20. Collect the DNA by centrifugation at 25,000*g* for 30 minutes at 0°C. Carefully decant the ethanol, and wash the pellet of DNA with several milliliters of 70% ethanol at room temperature. Again, recover the DNA by centrifugation, and remove the 70% ethanol by careful aspiration. Stand the open tube on the bench until the last traces of ethanol have evaporated. Dissolve the DNA in 20 μl of TE (pH 7.6), and estimate the recovery of DNA by analyzing a small aliquot by electrophoresis through an agarose gel.

21. Use approximately 50 ng of the DNA to transform competent *E. coli* (strain DH1 or DH5) to resistance to ampicillin *only*. Plate the transformants on LB agar medium containing ampicillin (100 μg/ml), and incubate them for 18–24 hours at 37°C. Store the remainders of the ligation mixtures at −20°C.

22. Pick 36 independent transformants, and establish small-scale (2-ml) cultures in LB medium containing ampicillin (100 μg/ml). After the

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cultures have reached saturation, isolate the plasmid DNA using one of the methods described in Chapter 1. Analyze the DNAs by digestion with the appropriate restriction enzymes and gel electrophoresis to obtain an estimate of the proportion of clones that carry a linker in the target region.

23. Screen the transformed colonies until two independent isolates have been obtained that carry the hexameric linker at each site that is cleaved by the restriction enzyme used originally to linearize the plasmid DNA (step 4).

24. Verify by DNA sequencing that the linkers have been inserted cleanly and that no deletion or addition of nucleotides has occurred at the site of insertion.

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CREATING MANY MUTATIONS IN A DEFINED SEGMENT OF DNA

At present, it is impossible to predict with accuracy the effect of substituting one amino acid for another in a protein. Current attempts to "improve" the properties of a protein therefore depend on analyzing large numbers of variants that are created by site-directed mutagenesis in promising regions (e.g., in and around the active site of an enzyme). Clearly, the number of potential variations that can be created, even in a circumscribed region of a protein, is extremely large. For example, 114 different mutants would be required simply to insert every possible amino acid at each of six locations in a protein. This number grows to 6^{19} if such substitutions are made in a combinatorial fashion. When planning this type of mutagenesis, careful choices must therefore be made to keep the numbers of mutants within manageable limits. For example, the numbers of potential mutants can be markedly reduced by avoiding replacements that are (1) highly conservative (i.e., the substitution of one amino acid with another whose chemical properties are very similar), (2) highly radical (i.e., replacing an amino acid with another whose chemical properties are completely different), or (3) misguided (e.g., the substitution of cysteine residues in secretory proteins). However, when the number of desired mutants exceeds 20 or so, it becomes impractical and expensive to generate each of them individually using a separate mutagenic oligonucleotide. Methods have therefore been devised to use degenerate pools of oligonucleotides to create large populations of mutants in a single round of site-directed mutagenesis. These degenerate pools of oligonucleotides contain a mixture of normal and abnormal bases at each position in the sequence at which a mutagenic event is desired. In the remainder of this section, we present guidelines for ways in which these populations of clustered mutations can be efficiently generated using degenerate pools of mutagenic oligonucleotides.

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Use of Degenerate Pools of Mutagenic Oligonucleotides

1. Pools of degenerate single-stranded oligonucleotides can be used only when the target amino acids are clustered. If all of the codons that are to be altered lie within a short stretch of contiguous nucleotides, a pool of degenerate single-stranded mutagenic oligonucleotides can be used as mismatched primers on single-stranded DNA templates to generate the corresponding set of mutants. However, the mutants cannot generally be distinguished from the original wild-type DNA by the standard method of screening by hybridization. In most cases, the mutagenic oligonucleotides are so long that there is no practical difference in stability between mismatched and perfect hybrids. Even if the oligonucleotides are sufficiently short (≤ 20 nucleotides in length), the pool usually contains many different members, each of which has different hybridization characteristics. It is therefore extremely difficult, if not impossible, to devise hybridization conditions that will distinguish all possible mutant sequences from the original wild-type sequences. This type of mutagenesis is therefore best carried out using the Kunkel system (see pages 15.74–15.79), which selects strongly against bacteriophages generated by replication of the original wild-type (+) strand of DNA. Mutants are then identified by picking individual plaques blindly and sequencing the relevant section of single-stranded bacteriophage DNA.

2. An alternative method is to generate pools of mutants by "cassette mutagenesis," a technique that involves replacing the wild-type sequence with synthetic double-stranded oligonucleotides (see, e.g., McNeil and Smith 1985; Wells et al. 1985; Derbyshire et al. 1986; Hill et al. 1986, 1987; Hutchison et al. 1986; Bedwell et al. 1989). Since cassette mutagenesis was first introduced (Matteucci and Heyneker 1983), several variations have been described, each of which has advantages under particular circumstances. However, all of these techniques suffer from a common drawback—the necessity for unique restriction sites at both ends of the cassette. Because these restriction sites are required to shuttle the synthetic double-stranded oligonucleotide into the correct location, they cannot occur anywhere else in either the plasmid vector or the segment of the wild-type gene that it carries. Furthermore, to ensure that the cassette is inserted in the correct orientation, the cassette should carry different restriction sites at each end. Because naturally occurring restriction sites hardly ever fulfill these criteria, it is usually necessary to carry out one or more rounds of site-directed mutagenesis to create suitable restriction sites at the appropriate locations in the wild-type gene. If the introduction of these sites changes the amino acid sequence encoded by the gene, it is necessary to determine whether the resulting protein displays wild-type characteristics. To eliminate the possibility that the phenotypes of any mutants obtained by cassette mutagenesis result from a combination of amino acid changes (i.e., changes caused by introduction of the restriction sites and by changes encoded within the cassette), it may be necessary to restore the original wild-type sequence at the restriction sites.

   Three different methods are currently used to generate double-stranded

oligonucleotide cassettes. In the first method (McNeil and Smith 1985) (see Figure 15.9A), two separate sets of oligonucleotides are synthesized that are complementary to the opposite strands of the target DNA. One of these sets consists of a single species of oligonucleotide that is exactly complementary to the sequence of one of the strands of the wild-type target DNA. The other set consists of a degenerate pool of oligonucleotides that is complementary to the opposite strand and that carries the desired mutations. These sets of complementary oligonucleotides are then mixed under conditions that will allow mismatched hybrids to form. If the complementary oligonucleotides have been designed to yield double-stranded cassettes that carry the appropriate protruding termini, they can be inserted directly into a recombinant plasmid in place of the homologous wild-type sequence. Alternatively, cohesive termini can be created by digesting double-stranded blunt-ended cassettes with the appropriate restriction enzymes. The mismatches in the recombinant plasmids are repaired in vivo, after the recombinant plasmids have been introduced into competent bacteria. Subsequent replication of the plasmid DNA and segregation into daughter cells allows clones to be isolated that are derived from each DNA strand of the plasmid originally used for transformation. In this method and the one that follows, the plasmids isolated from individual colonies of transformed bacteria are occasionally heterogeneous, suggesting that segregation of the plasmids is sometimes incomplete. This problem can be solved by retransforming competent bacteria with plasmid DNAs extracted from pooled primary transformants. However, in this first method of cassette mutagenesis, the frequency of mutation can never exceed 50% because only one half of the progeny plasmids are derived from the mutagenized strand.

In the second method (see Figure 15.9B), the frequency of mutation is increased by using cassettes in which the complementary strands both consist of mixed-sequence oligonucleotides (Wells et al. 1985). Because each of these strands gives rise to progeny plasmids, the mutation rate can be raised to greater than 50% (Makris et al. 1988).

In the third method (see Figure 15.9C), degenerate pools of single-stranded oligonucleotides are converted to a blunt-ended double-stranded form by mutually primed synthesis (Oliphant et al. 1986; Hill et al. 1987). Two degenerate pools of oligonucleotides are synthesized that are complementary to the same strand of the target DNA. However, the members of one pool carry sequences at their 3′ termini that are complementary to sequences at the 3′ termini of oligonucleotides in the second pool. Usually, these complementary sequences are palindromic and correspond to the restriction site that marks one end of the cassette. The oligonucleotides in the two pools are then annealed to form partial hybrids that can be converted to blunt-ended double-stranded DNA by the Klenow fragment of *E. coli* DNA polymerase I. The products of this reaction are tail-to-tail dimers. Unit-length cassettes are generated by digesting the dimers with the appropriate restriction enzymes.

The major advantage of the third method is that the unit-length cassette consists of perfect homoduplexes. Any potential bias that occurs during mismatch repair in vivo is therefore avoided, and there is no loss of

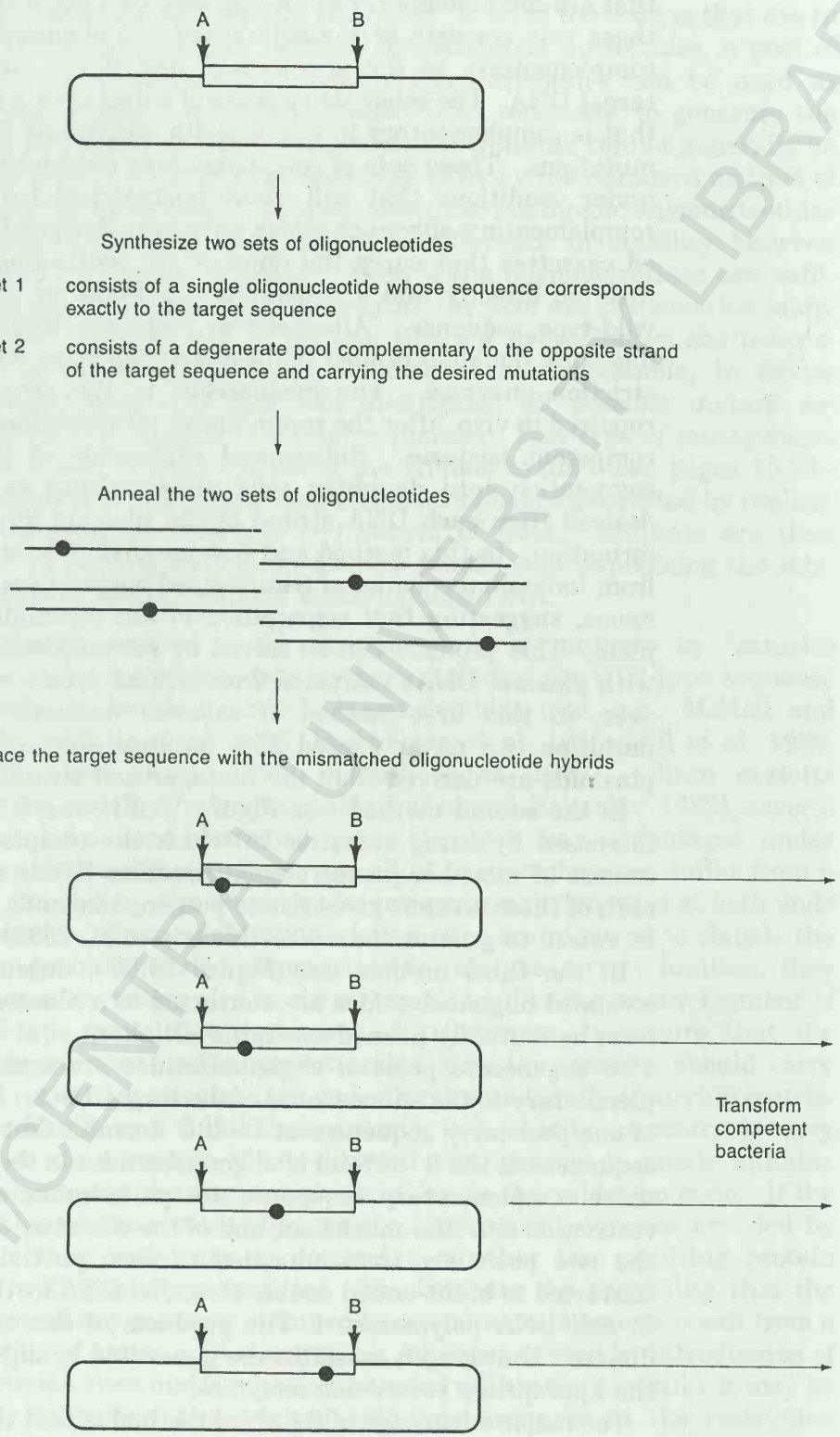
A
B
Synthesize two sets of oligonucleotides
Set 1 consists of a single oligonucleotide whose sequence corresponds exactly to the target sequence
Set 2 consists of a degenerate pool complementary to the opposite strand of the target sequence and carrying the desired mutations
Anneal the two sets of oligonucleotides
Replace the target sequence with the mismatched oligonucleotide hybrids
Transform competent bacteria

FIGURE 15.9A
Cassette mutagenesis using a single mixed-sequence oligonucleotide and repair of mismatches in vivo.

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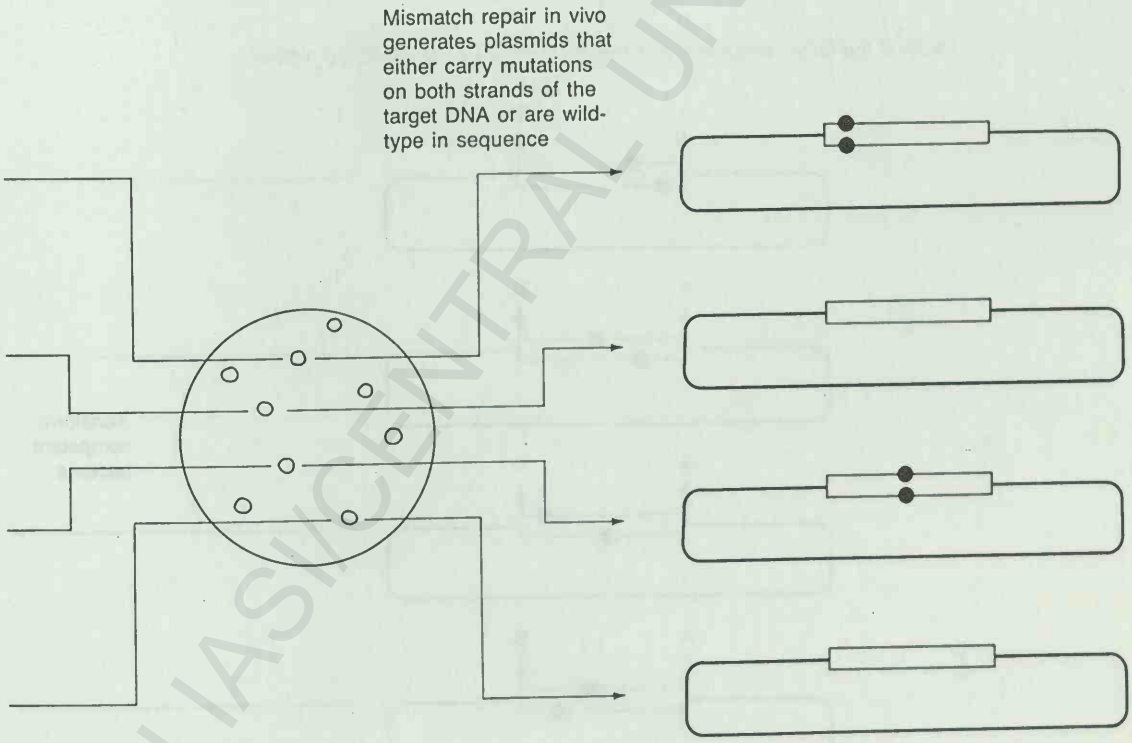
Mismatch repair in vivo generates plasmids that either carry mutations on both strands of the target DNA or are wild-type in sequence

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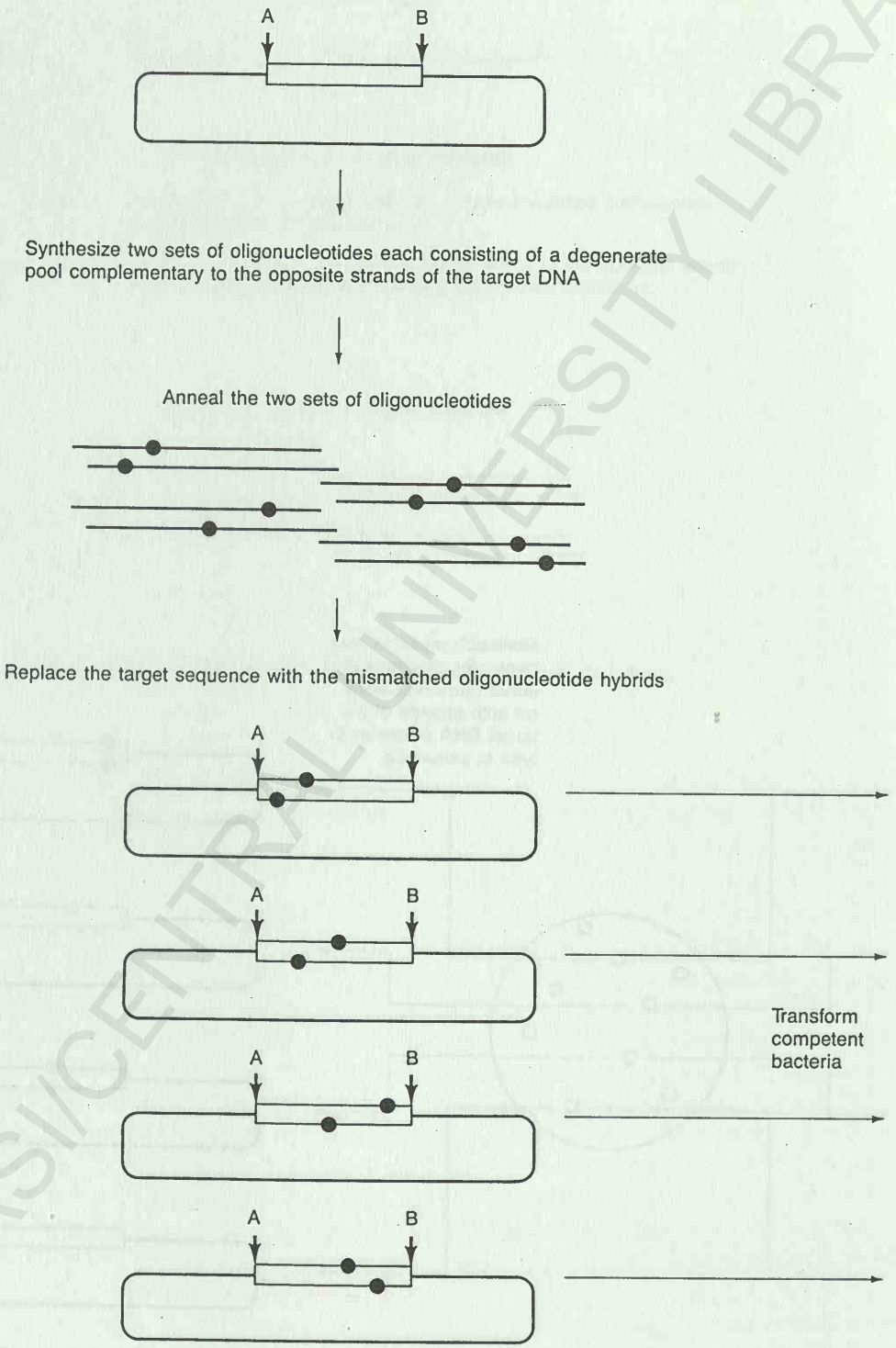

FIGURE 15.9B
Cassette mutagenesis using two complementary mixed-sequence oligonucleotides and repair of mismatches in vivo.

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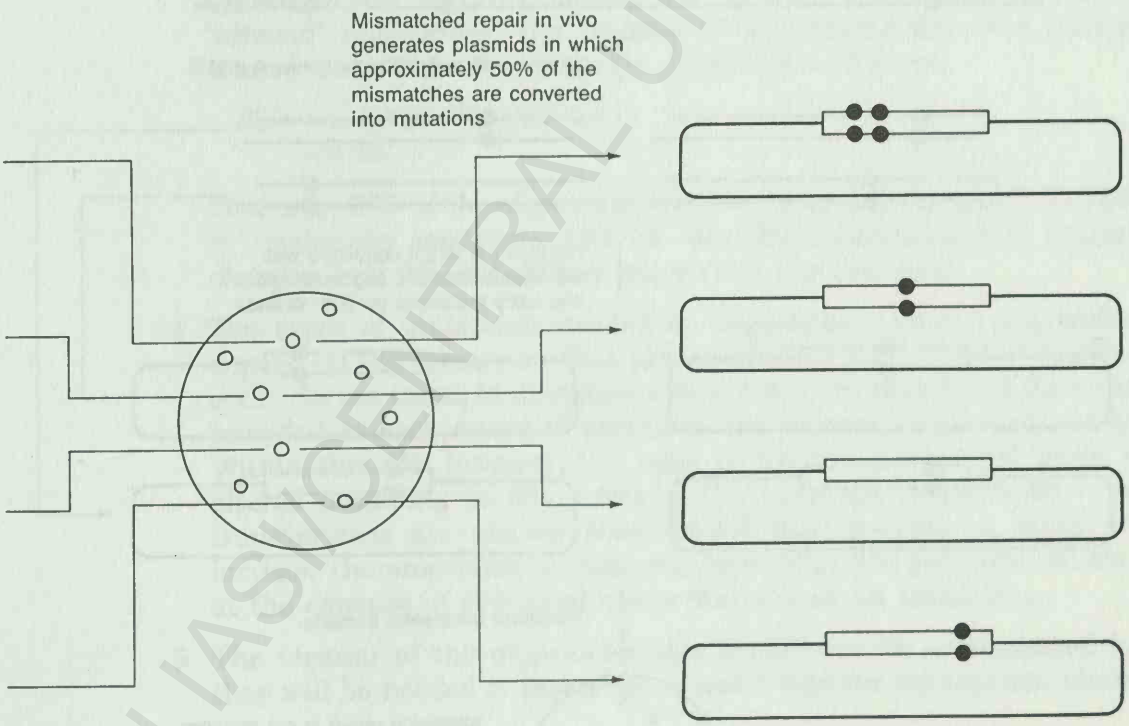

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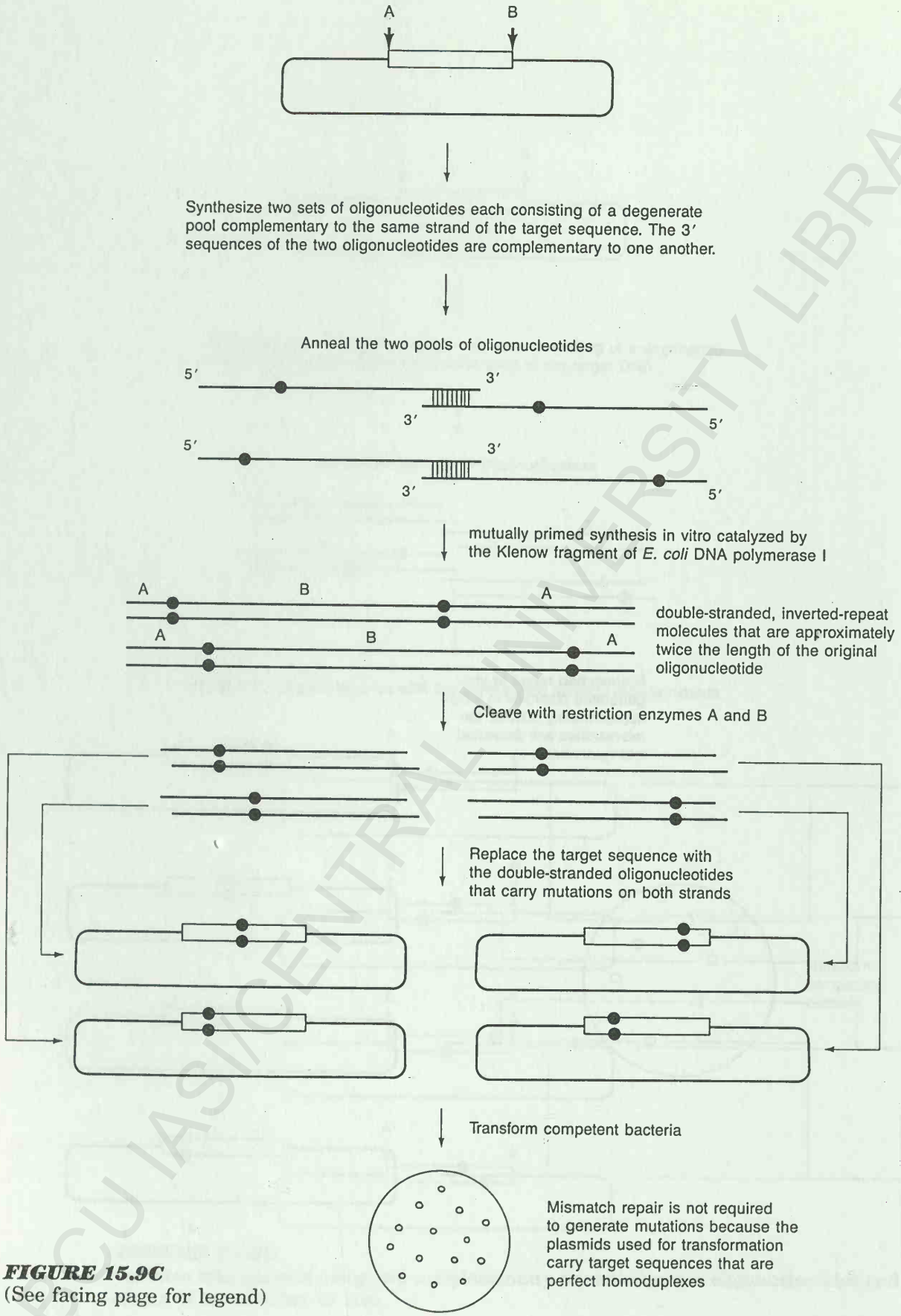
A
B
Synthesize two sets of oligonucleotides each consisting of a degenerate pool complementary to the same strand of the target sequence. The 3′ sequences of the two oligonucleotides are complementary to one another.
Anneal the two pools of oligonucleotides
5′
3′
3′
5′
5′
3′
3′
5′
mutually primed synthesis in vitro catalyzed by the Klenow fragment of E. coli DNA polymerase I
A
B
A
A
B
A
double-stranded, inverted-repeat molecules that are approximately twice the length of the original oligonucleotide
Cleave with restriction enzymes A and B
Replace the target sequence with the double-stranded oligonucleotides that carry mutations on both strands
Transform competent bacteria
Mismatch repair is not required to generate mutations because the plasmids used for transformation carry target sequences that are perfect homoduplexes

FIGURE 15.9C
(See facing page for legend)

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mutants because of correction to wild-type sequences. Finally, because segregation is not required, primary bacterial transformants contain pure plasmid populations that can be analyzed directly. For these reasons, this is currently the method of choice for creating mutations at many sites within a defined region of DNA.

3. Ideally, each member of a degenerate pool of oligonucleotides should contain one nucleotide change per target sequence. In practice, however, because the oligonucleotide pools are generated in a mixed synthetic reaction, the best that can be achieved is an *average* of one altered base per target sequence. At each cycle, therefore, there is a chance that either a normal or an altered base will be incorporated into a growing oligonucleotide chain. The mutation frequency at any given site depends on the relative concentrations of the different nucleotide precursors that are provided at a particular cycle in the synthetic reaction.

   The fraction of oligonucleotides that contain nucleotide changes follows a binomial distribution that can be predicted from the following equation (McNeil and Smith 1985; Makris et al. 1988):

   $$F(P) = n!P^{n-r}(1-P)^{r}/(n-r)!r!$$

   where $F(P)$ is the fraction of the population whose sequence contains r random base changes over a target sequence of n consecutive bases, P is the probability of any given nucleotide being unchanged, and $(1-P)$ is the probability of any given nucleotide being changed. For example, when the length of the target sequence is 20 and the mixture of precursors supplied at every cycle contains 95% of the "normal" nucleotides and 5% of the "altered" nucleotides, the fraction of oligonucleotides that contain one altered nucleotide ($r = 1$) can be calculated as follows:

   $$F(P) = 20!(0.95)^{19}(0.05)^{1}/19!1!$$
   $$= 0.38$$

   Similarly, 36% of the oligonucleotides in the pool will contain no alteration in nucleotide sequence; 19% of the oligonucleotides will contain two changes, and 7% will contain more than two changes.

4. The types of mutations created by degenerate pools of oligonucleotides depend on the precursors that are supplied at each round of the synthetic cycle. In the example discussed above, not more than 5% of the precursors provided at each round of synthesis can contain "abnormal" nucleotides. Within this 5%, however, the ratio of the three abnormal bases can be altered according to the needs of the particular experiment. Because transversions are usually more useful than transitions, many workers increase the proportion of abnormal bases that will generate transversions at the expense of abnormal bases that will cause transitions.

5. The termini of the oligonucleotides should not be mutagenized because they will be needed to insert the cassette into the appropriate plasmid. If

FIGURE 15.9C
Cassette mutagenesis using two partially overlapping mixed-sequence oligonucleotides and complementary strand synthesis in vitro.

cohesive termini are to be generated by cleaving the double-stranded cassettes with restriction enzymes, three extra nucleotides should be added to each end of the mutagenic oligonucleotide. These extensions increase the efficiency of digestion with restriction enzymes.

6. The frequency with which mutants are obtained at any particular position decreases as the length of the mutagenic oligonucleotides in the degenerate pool increases. Because individual mutants are recovered by random sampling, it is improbable that all possible mutations will be isolated when the size of the potential pool is large. Under these circumstances, "missing" clones that carry particularly interesting mutations can usually be identified by hybridization to specifically designed oligonucleotide probes.

Finally, it is worth remembering that oligonucleotide-mediated mutagenesis is not the only method that can be used to saturate segments of cloned DNA with mutations. Several of the other techniques that are available are discussed below.

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Treatment of Double-stranded DNA with Chemical Mutagens

The simplest method of localized random mutagenesis is to react a short fragment of double-stranded DNA with a chemical mutagen such as nitrous acid or hydroxylamine and to clone the population of mutagenized fragments into a recombinant plasmid that carries the remainder of the wild-type gene. Recombinant plasmids carrying mutations that generate a novel phenotype can be recognized by appropriate tests. For example, a temperature-sensitive mutation constructed in a gene coding for a mammalian protein might be recognized by immunofluorescent staining of mammalian cells that had been transfected with the appropriate plasmid incubated at permissive and non-permissive temperatures. Recombinant plasmids carrying a mutation that does not give rise to an easily assayed phenotype must be identified by DNA sequencing of random clones. Unfortunately, the frequency at which mutants are recovered by this method is unacceptedly low (Chu et al. 1979; Solnick 1981; Busby et al. 1982; Kadonaga and Knowles 1985). Furthermore, because chemical mutagens react with bases in double-stranded DNA in a highly specific manner, only a limited spectrum of mutations is recovered. For these reasons, this method is no longer in widespread use.

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Treatment of Single-stranded DNA with Sodium Bisulfite

In the original descriptions of this protocol, circular double-stranded plasmid DNA was nicked at a random site with pancreatic DNAase I in the presence of ethidium bromide (Greenfield et al. 1975; Shortle and Botstein 1983). The nick was then converted to a gap by digestion with exonuclease III, and the resulting gapped double-stranded molecule was exposed at slightly acid pH to sodium bisulfite (1–3 M), which caused deamination of cytosine to uracil. After transformation of bacteria, replication of the mutagenized DNA led to replacement of the original C:G base pair with a T:A base pair. Recently, the efficiency of this type of mutagenesis has been improved by carrying out deamination on gapped duplexes of bacteriophage M13 recombinants in which the target DNA is exposed in a single-stranded form (Pine and Huang 1987). After mutagenesis, the DNA is transfected into an *ung*$^-$ strain of *E. coli* that is unable to remove the newly generated uracil residues. Although the procedure results in highly efficient mutagenesis of a defined segment of DNA, it generates only transition mutations in which a purine replaces a purine on one strand of DNA and a pyrimidine replaces a pyrimidine on the other. Unfortunately, mutations of this type generally result in conservative substitutions of amino acids. Thus, the range of mutants that are obtained is often too narrow to allow a comprehensive analysis of a particular segment of a protein (Shortle and Nathans 1978; DiMaio and Nathans 1980; Peden and Nathans 1982).

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Treatment of Single-stranded DNA with Chemicals That Damage All Four Bases

In this method (Myers et al. 1985a), single-stranded DNA of a recombinant M13 bacteriophage is exposed under defined conditions to chemicals (nitrous acid, formic acid, and hydrazine) that modify bases in single-stranded DNA without breaking the phosphodiester backbone (see Chapter 13). After removal of the chemicals, a universal sequencing primer and avian reverse transcriptase are used to synthesize the complementary strand of DNA. When the polymerase encounters damaged bases in the template strand, it incorporates nucleotides essentially at random. Because all possible nucleotides can be incorporated at a single position, there is a 75% probability of mutation at every site of damage. Furthermore, because tranversions are generated twice as frequently as transitions, the resulting mutations generate proteins with a wide spectrum of amino acid changes. After the extension reaction is completed, the double-stranded target fragment is excised and recloned into an appropriate vector. Mutants can be identified directly by DNA sequencing of random clones.

The major problem with this method is the frequency with which useful mutations can be isolated. To prevent the formation of unacceptable numbers of multiple mutants, it is necessary to limit carefully the length of time the single-stranded DNA is exposed to damaging chemicals. However, this means that many of the template strands escape modification altogether. Therefore, the best that can be achieved by this method is a frequency of single mutations of 10–15%. This problem can sometimes be alleviated by using denaturing gradient gel electrophoresis to purify fragments of DNA that carry mutations (Myers et al. 1985a,b). However, this technique is by no means simple, and it requires the attachment of the mutagenized DNA to special vectors equipped with GC clamps (Myers et al. 1985c). Because of these problems, this method of mutagenesis has so far not found widespread acceptance.

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Misincorporation of Nucleotides by DNA Polymerases

Point mutations can be introduced into double-stranded DNA by incorporating base analogs with various types of DNA polymerases. For example, Shortle and his coworkers (Shortle et al. 1982; Shortle and Lin 1985) incubated gapped DNA in the presence of *E. coli* DNA polymerase I and only one of the four α-thiophosphate dNTPs. Thiophosphate dNTPs are efficiently incorporated by the polymerase but are not effectively removed by its $3' \rightarrow 5'$ editing function. The incorrect base is thus incorporated at a high frequency, and the remainder of the gap is then filled in a second polymerization reaction carried out in the presence of all four of the normal dNTPs. All types of base substitutions have been obtained with this method using each of the four α-thiophosphate dNTPs in separate repair reactions.

Another misincorporation method uses AMV reverse transcriptase, which is deficient in a $3' \rightarrow 5'$ exonuclease activity (Zakour and Loeb 1982). In this case, conventional dNTPs are used to synthesize DNA from an upstream primer. Base analogs are then incorporated in the region of interest.

The major problem of these and other misincorporation methods is the difficulty in creating populations of template molecules in which the 3′ hydroxyl terminus of the growing strand is located at random positions throughout the region of interest. Although this can in theory be achieved by a number of different methods (e.g., controlled nick translation with *E. coli* DNA polymerase I or controlled digestion of double-stranded DNA with exonuclease III), the routine generation of large numbers of mutations at random sites has proved to be difficult in practice. Success requires careful characterization of the reagents involved, meticulous establishment of optimal reaction conditions, and many trial experiments.

In summary, whereas methods (discussed earlier in this chapter) to introduce single mutations in cloned DNA are now well-established, techniques to saturate defined regions with mutations are less satisfactory. Using chemical mutagenesis, the rate of production of single mutations is low and/or the mutations themselves are of limited interest. Using misincorporation of base analogs, it is difficult to direct the mutations to the region of interest. However, it seems likely that at least some of these problems will be solved during the next few years, for example, by incorporating base changes into DNA synthesized in polymerase chain reactions or by advances in DNA chemistry that will facilitate the synthesis of mutagenized DNAs of extended length. Until then, we recommend using degenerate pools of synthetic oligonucleotides. In contrast to the other methods, the mutations can be precisely designed by the experimenter and can be focused in a defined region of DNA. Even with these limitations, the amount of work involved in isolating and characterizing a comprehensive set of mutants remains very large. In this branch of molecular cloning, therefore, it is especially important to weigh the potential scientific rewards against the commitment of time and personnel that the project will certainly consume.

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